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Qualitative Determination of Specific Protein Glycation Products by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Peptide Mapping

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The nonenzymatic reaction between reducing sugars and proteins, known as the Maillard reaction, has received increased recognition from nutritional science and medical research. The development of new analytical techniques for the detection of protein-bound Maillard products is therefore crucial. In this study, we applied peptide mapping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to investigate the formation of structurally specific Maillard products on glycated lysozyme (AGE-lysozyme), produced upon incubation with D-glucose. In parallel, we synthesized *N*-(carboxymethyl)lysine-modified lysozyme (CML-lysozyme) and *N*-(carboxyethyl)lysine-modified lysozyme, two well-described glycation products, as model substances. 3-Deoxyglucosone-modified lysozyme and methylglyoxal-modified lysozyme were prepared as examples of glycation products incubated with dicarbonyl compounds. We were able to detect specific modifications on AGE-lysozyme, which were assigned to CML, imidazolone A, and the Amadori product.

KEYWORDS: Maillard reaction; MALDI-TOF-MS peptide mapping; protein glycation; *N*-(carboxymethyl)-lysine; imidazolone A

INTRODUCTION

The Maillard reaction, the nonenzymatic interaction between reducing sugars and amino groups, is one of the most important reactions in food. The reaction occurs during heat treatment or storage for long periods (1–3). The effects of nonenzymatic glycation on food proteins may be beneficial or undesirable, depending on the extent of glycation. Low extensions of protein glycation have been reported to improve their emulsifying properties (4–7), gelation, water-holding capacity, foaming, and solubility properties (8–12). On the other hand, high extensions of protein glycation result in protein cross-linking and loss of protein solubility (13). Importantly, extensive glycation is associated with the loss of nutritional value of the food proteins, due to the unavailability of the essential amino acid lysine after glycation (14), as well as the loss of protein digestibility (15, 16). In the last 15 years, it has become evident that the Maillard reaction also occurs in vivo, where it could be linked to the pathogenesis of age-related diseases, such as diabetes mellitus, atherosclerosis, and Alzheimer's. In this context, a large number of studies have suggested a potential role of advanced glycation

endproducts (AGEs) in these age-related diseases (17–20). Several of these in vivo-occurring late stage Maillard products or AGEs have been structurally identified (21–28). Furthermore, several of these structurally defined glycation products have been detected in food products in recent years (29, 30).

Common methods for the analysis of protein glycation products include immunochemical methods (31, 32) such as immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and Western blotting, using antibodies specific for certain AGE structures, or chromatographic techniques such as gas chromatography/mass spectrometry (GC/MS) (33, 34), high-performance liquid chromatography (HPLC/MS) (35), and HPLC with fluorescence detection (36, 37) after hydrolysis of the protein. However, immunochemical methods are restricted to detection and quantitation of marker compounds for glycation, whereas the drawback of chromatographic methods lies in high detection limits as compared to low modification rates found. Furthermore, the requirement of a sufficient hydrolysis step that is, nevertheless, mild enough to prevent artifact formation limits the use of GC or HPLC. At the same time, protein glycation products in vivo and in food are a very heterogeneous mixture of different products. Therefore, it can be assumed that the majority of protein glycation structures has not yet been identified. It is also not clear which products are formed in the

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highest yields in vivo or in food and are of physiological importance.

With the introduction of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) (38, 39), the application of MS to large biomolecules has been revolutionized. As the glycation of lysozyme increases its molecular weight, we turned to MALDI-TOF-MS to determine protein modifications formed by the Maillard reaction. Previous reports indicated the usefulness of MALDI-TOF-MS for the detection of protein glycation products in vivo and in vitro (40–48). All of these authors used MALDI-TOF-MS to determine a mass increase of intact, glycated proteins. To resolve the modifications to a structural level, molecular mapping of the modification sites is required. For this reason, we performed MALDI-TOF-MS after peptide mapping of glycated lysozyme, serving as a model protein. This method provided fragments of significantly lower molecular mass, permitting a residue specific resolving of glycation structures formed by the Maillard reaction. As a result, we are able to introduce MALDI-TOF-MS peptide mapping as a valuable technique for the detection of specific products formed by the nonenzymatic glycation of lysozyme.

MATERIALS AND METHODS

Chemicals. Lysozyme (chicken egg), glyoxylic acid, pyruvic acid, methylglyoxal, D-glucose, NaCNBH₃, sodium phosphate, 3,5-dimethoxy-4-hydroxycinnamic acid, α -cyano-4-hydroxycinnamic acid, TRIS base, sodium dodecyl sulfate, dithiothreitol (DTT), glycine, and TRICINE were purchased from Sigma-Aldrich (Deisenhofen, Germany). Endoproteinase Glu-C was purchased from Roche Diagnostics (Mannheim, Germany). Precast 16.5% peptide gels (Criterion) were purchased from Bio-Rad (Munich, Germany).

Synthesis of Defined AGE Proteins. N^ε-(Carboxymethyl)lysine-modified lysozyme (CML-lysozyme) was prepared as described previously (49). Briefly, lysozyme (100 mg) was incubated at 37 °C for 24 h with 3 mg of glyoxylic acid and 10 mg of NaCNBH₃ in 10 mL of 0.2 M sodium phosphate buffer (pH 7.8), followed by dialysis against doubly distilled water and lyophilization. N^ε-(Carboxyethyl)lysine-modified lysozyme (CEL-lysozyme) was prepared by incubating 100 mg of lysozyme at room temperature for various times with 15 mg of pyruvic acid and 20 mg of NaCNBH₃ in 10 mL of 0.2 M sodium phosphate buffer (pH 7.8), followed by dialysis against doubly distilled water and lyophilization. To prepare CEL-lysozyme with lower modification rate, 100 mg of lysozyme was incubated with pyruvic acid (4 mg) and NaCNBH₃ (10 mg) in the same way as described.

In Vitro Glycation of Lysozyme. 3-Deoxyglucosone-modified lysozyme (3-DG lysozyme) was prepared by incubation of 100 mg of lysozyme with 10 mg of 3-deoxyglucosone in 10 mL of 0.2 M sodium phosphate buffer (pH 7.8) at 37 °C for 4 days, followed by dialysis against doubly distilled water and lyophilization. Methylglyoxal-modified lysozyme (MG-lysozyme), in different modification degrees, was prepared by incubation of 100 mg of lysozyme with methylglyoxal, 40% aqueous solution (25, 10, 5, and 1 mM and 500 μ M), in 10 mL of 0.2 M sodium phosphate buffer (pH 7.8). Samples were taken after 4, 22, and 30 h and dialyzed against doubly distilled water followed by lyophilization. AGE-lysozyme was prepared as described previously (50). Briefly, lysozyme (100 mg) was dissolved with different amounts of D-glucose (500, 100, 50, and 25 mM) in 10 mL of phosphate-buffered saline (PBS, pH 7.8) and incubated for 3 weeks at 50 °C or for 8 weeks at 37 °C, followed by dialysis against doubly distilled water and lyophilization. The incubation mixtures were sterile-filtered prior to incubation.

ELISA Procedure. A noncompetitive ELISA was performed as previously described (29). Briefly, microtiter plates were coated overnight at 4 °C with 200 μ L of a solution of CML-lysozyme, AGE-lysozyme, or native lysozyme in 50 mM carbonate buffer (pH 9.7) in a concentration range between 1 ng/mL and 1 mg/mL. After each step, the plates were washed twice with PBS containing 0.05% Tween 20. The coated plates were blocked with 300 μ L of a 3% solution of nonfat

milk powder in PBS for 2 h at room temperature while shaking. Then, 200 μ L of CML specific antiserum (dilution 1:25 000 in PBS containing 0.2% bovine serum albumin (BSA) and 0.05% Tween 20) was added, and the plates were shaken for 1 h at room temperature. In the development step, the wells were incubated for 45 min with 200 μ L goat antirabbit IgG alkaline phosphatase conjugate (diluted 1:10 000 in 0.1% BSA in PBS) and washed three times with PBS and 0.05% Tween 20. Antibody binding was detected using 150 μ L of tetramethylbenzidine solution. The reaction was stopped after 15 min by adding 50 μ L of 2 N sulfuric acid.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE) Peptide Gels. Precast peptide gels were used to separate native lysozyme, synthetic, defined AGE proteins, and glycated lysozyme. Protein samples (4 μ g) were electrophoretically separated at 120 V, using a TRIS/TRICINE/SDS buffer, followed by Coomassie staining.

Endoproteinase Glu-C Digestions. Lysozyme and modified lysozymes were dissolved in 25 mM sodium phosphate buffer (pH 7.8) to a final concentration of 1 nmol/ μ L. For the digestion reaction, 5 μ L of the lysozyme solutions, 3 μ L of endoproteinase Glu-C (3 μ g), and 2 μ L of water were incubated at 25 °C for 15 h. Prior to MS, 1 μ L of 100 mM DTT solution was added. In 25 mM sodium phosphate buffer (pH 7.8), endoproteinase Glu-C specifically cleaves peptide bonds C-terminal to the amino acids glutamic acid and aspartic acid (according to the manufacturer's instructions).

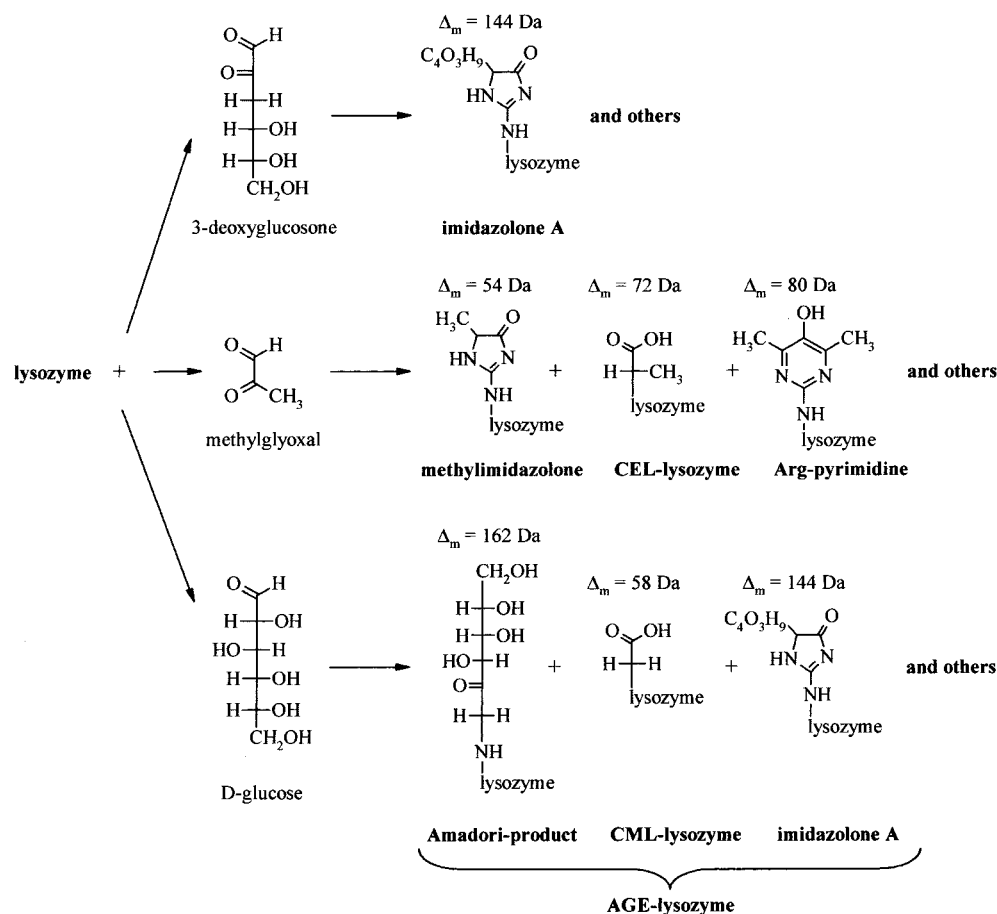
MALDI-TOF-MS. For analysis by MALDI-TOF-MS, samples (500 pmol/ μ L) were diluted 1:50 in a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (intact protein) or α -cyano-4-hydroxycinnamic acid (peptide digest) in 0.1% trifluoroacetyl with 33% acetonitrile. An aliquot (1 μ L) of this mixture was spotted onto a stainless steel target, air-dried, and subjected to mass determination using a Biflex III MALDI-TOF-MS (Bruker Daltonik, Bremen, Germany). The instrument was equipped with a nitrogen laser (λ 337 nm) and a reflector. Measurements were performed using delayed extraction. Laser-desorbed positive ions were analyzed after acceleration by 20 kV in the linear mode for the intact protein and by 19 kV in the reflector mode for the peptide digest. External calibration was performed by use of a standard peptide/protein mixture. Usually, 30 individual spectra were averaged to produce a mass spectrum.

RESULTS

We attempted the simultaneous identification of different structurally defined protein glycation products on heterogeneously glycated lysozyme (AGE-lysozyme). Therefore, we synthesized CML-lysozyme and CEL-lysozyme, two well-described AGE structures, as model substances. Furthermore, 3-DG-lysozyme and MG-lysozyme were prepared as examples for proteins modified by reactive dicarbonyl compounds. Using these substances as references, we analyzed spectra of AGE-lysozyme, produced upon incubation of lysozyme with different amounts of D-glucose at 37 or 50 °C in PBS (**Scheme 1**).

Modifications of CML-lysozyme were confirmed by ELISA, using a polyclonal antibody specific for CML modifications. AGE-lysozyme produced upon incubation of lysozyme with D-glucose also showed CML modification, as detectable by the polyclonal antibody described above (data not shown).

SDS–PAGE Peptide Electrophoresis. SDS–PAGE is a method commonly used to separate unfragmented, glycated proteins (51). Initially, lysozyme and in vitro modified lysozymes were electrophoretically separated on a 16.5% SDS–PAGE peptide gel (data not shown). Only high-modified AGE-lysozyme, produced upon incubation of lysozyme with 500 mM D-glucose, showed a mass increase large enough to be separated by SDS–PAGE electrophoresis. In contrast, neither low-modified AGE-lysozyme, produced upon incubation of lysozyme with 50 mM D-glucose, nor any of the other lysozyme modifications could be separated from the native protein using

Scheme 1. In Vitro Glycation of Lysozyme^a

^a Reaction of lysozyme with D-glucose and reactive sugar degradation products (3-deoxyglucosone and methylglyoxal) is indicated. Structures of identified reaction products and Δm are presented.

SDS-PAGE. High-modified AGE-lysozyme as well as MG-lysozyme clearly showed a band broadening, as compared to native lysozyme, due to the heterogeneity of the resulting products.

MALDI-TOF-MS Analysis of Intact Proteins. As accuracy of molecular mass determination by SDS-PAGE is severely limited, we turned to MALDI-TOF-MS for the mass determination of intact, modified proteins (Figure 1). The molecular ion of native lysozyme produced a mass peak of m/z 14 313 (Figure 1A). In all of the modified lysozymes, at least one additional peak was observed. CML-lysozyme (Figure 1B) had one additional peak at m/z 14 371. The mass difference of 58 Da corresponded to carboxymethylation of the native protein. CEL-lysozyme (Figure 1C) had one additional peak at a molecular mass of m/z 14 385, with a mass difference of 72 Da resulting from carboxyethylation of native lysozyme. 3-DG-lysozyme (Figure 1D) yielded an additional peak at m/z 14 457. The molecular mass difference of 144 Da was very likely due to modification of an arginine residue with imidazolone A (Scheme 1). MG-lysozyme, produced upon incubation of lysozyme with 25 mM methylglyoxal at room temperature for 4 h (Figure 1E), showed a broad peak with the approximate maximum at m/z 15 000. This mass distribution was due to multiple modifications by methylglyoxal. The spectrum of glycated lysozyme (AGE-lysozyme) (Figure 1F) showed a very heterogeneous peak with a broad peak base. Several maxima were observed, with the main maximum at approximately m/z 15 000. The resulting mass difference was probably caused by multiple modification with the Amadori product, an early stage Maillard product, as well as post-Amadori modifications.

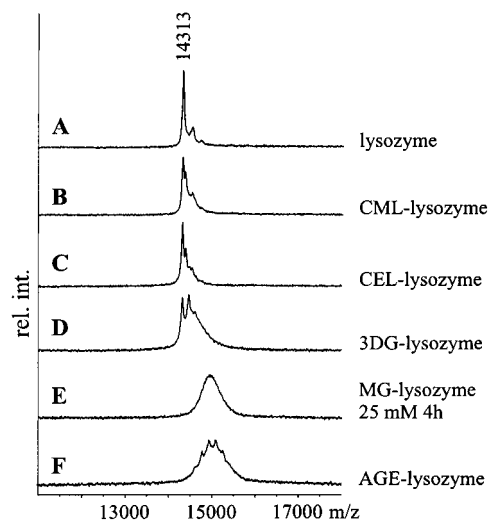


Figure 1. MALDI-TOF-MS spectra of intact proteins. m/z of main peaks is indicated.

However, specific modification products could not be resolved, as the mass increases introduced by specific glycation products were too small to be separated from the intact protein. Therefore, we applied peptide mapping to obtain more detailed structural information about the glycation products.

MALDI-TOF-MS Peptide Mapping. Native lysozyme and in vitro-modified lysozymes were enzymatically digested using endoproteinase Glu-C. The resulting peptide mixtures were subjected to MALDI-TOF-MS (Figures 2–6).

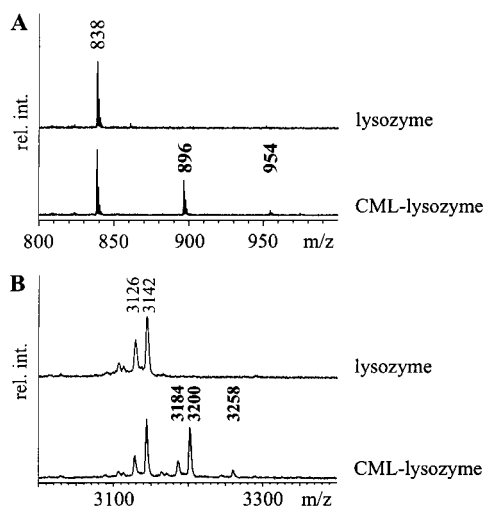


Figure 2. MALDI-TOF-MS peptide mapping analysis of CML-lysozyme. In panel **A**, the molecular weight range between m/z 800 and 1000 is presented, and in panel **B**, the molecular weight region between m/z 3000 and 3400 is presented. m/z of identified ions is indicated. New peaks are labeled in bold.

In **Figure 2**, a digest of carboxymethylated lysozyme was compared to a digest of native lysozyme. **Figure 2A** shows a spectrum of the region between m/z 800 and 1000, containing the N-terminal fragment (amino acids 1–7) with a molecular mass of m/z 838. The corresponding region of the CML-lysozyme contained two additional peaks at m/z 896 and 954. The mass differences of 58 and 116 Da resulted from single and double carboxymethylation, respectively. In the spectrum of native lysozyme, two molecular ions with masses of m/z 3126 and 3142 (amino acids 8–35) were observed (**Figure 2B**). In the equivalent spectrum of CML-lysozyme, three additional peaks corresponding to masses of m/z 3184, 3200, and 3258 had appeared. The ions with masses at m/z 3200 and 3258 corresponded to single and double carboxymethylation of the fragment at m/z 3142, whereas the fragment with a mass of m/z 3184 represents carboxymethylation of the ion at m/z 3126.

In **Figure 3**, CEL-lysozyme was compared to native lysozyme. Not surprisingly, the same regions as in CML-lysozyme were modified by carboxyethylation. In the spectrum of CEL-lysozyme, an additional peak at m/z 910 was observed as compared to native lysozyme (**Figure 3A**). The mass difference of 72 Da represented the increase theoretically expected for a carboxyethylated protein. The modification rate of the N-terminal fragment was dependent on incubation time and reactant concentrations (**Figure 3A**). **Figure 3B** represents a spectrum of the region between m/z 3000 and 3500. CEL-lysozyme clearly showed three new peaks at m/z 3198, 3214, and 3286, which were not present in the spectrum obtained from unmodified lysozyme. The peak with a mass of m/z 3198 corresponded to the carboxyethylated form of the ion at m/z 3126, whereas the peaks at m/z 3214 and 3286 resulted from single and double carboxyethylation, respectively, of the peak at m/z 3142. Again, different modification patterns dependent on incubation procedures were observed.

Next, 3-DG-lysozyme was compared to native lysozyme. One new peak was observed in the spectrum of 3-DG-lysozyme (**Figure 4A**). The molecular ion at m/z 1346 very likely resulted from a modification of the ion at m/z 1202 (amino acid 120–129) with imidazolone A. Because the fragment at m/z 1202 contains two arginine residues, the modification with imidazolone A seems very plausible. **Figure 4B** shows another region

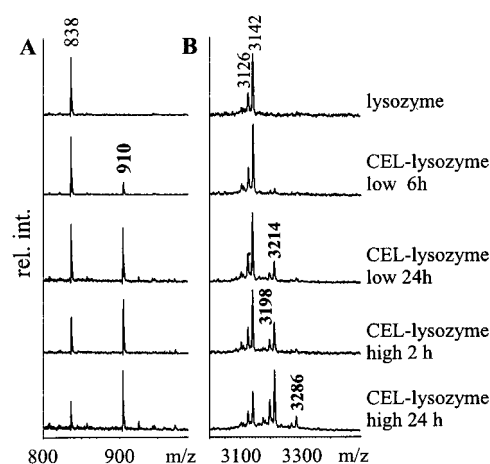


Figure 3. MALDI-TOF-MS peptide mapping analysis of CEL-lysozyme. In panel **A**, the molecular weight range between m/z 800 and 1000 is presented, and in panel **B**, the molecular weight region between m/z 3000 and 3400 is presented. m/z of identified ions is indicated. In panels **A** and **B**, detection of different low and high modification patterns, dependent on incubation procedure is shown. The incubation times are indicated in the figure. New peaks are labeled in bold.

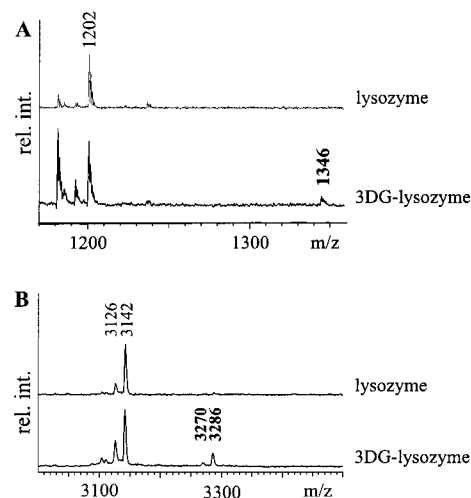


Figure 4. MALDI-TOF-MS peptide mapping analysis of 3-DG-lysozyme. In panel **A**, the molecular weight range between m/z 1170 and 1360 is presented, and in panel **B**, the molecular weight region between m/z 3000 and 3500 is presented. m/z of identified ions is indicated. New peaks are labeled in bold.

of interest in 3-DG-lysozyme. As compared to native lysozyme, two additional peaks with molecular masses of m/z 3270 and 3286 appeared in the spectrum of 3-DG-lysozyme. The molecular ion at m/z 3270 may result from modification of the ion at m/z 3126 and the ion at m/z 3286 from modification of the peak at m/z 3142. In each case, the observed mass difference was 144 Da, which is the theoretical mass increase for an imidazolone A modification.

In **Figure 5**, MG-lysozyme was compared to native lysozyme. In the spectrum of MG-lysozyme, several new ions appeared, three of which could be structurally assigned by mapping the Δm to a structurally known modification (**Figure 5A**). The ion at m/z 892 molecular mass very likely represented the methylimidazolone-modified form of the ion at m/z 838. The mass increase of 54 Da is the mass shift theoretically expected. The peak at m/z 910 could be the CEL-modified form of the ion at m/z 838. The same molecular mass had already been observed in CEL-lysozyme (**Figure 3A**). The peak at m/z 918 could be

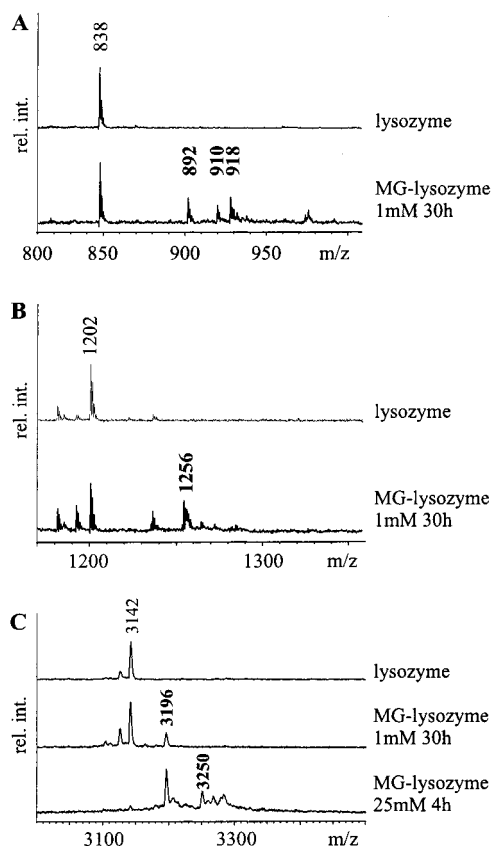


Figure 5. MALDI-TOF-MS peptide mapping analysis of MG-lysozyme. In panel A, the molecular weight range between m/z 800 and 1000 is presented, in panel B, the molecular weight range between m/z 1170 and 1360 is presented, and in panel C, the molecular weight region between m/z 3000 and 3400 is presented. m/z of identified ions is indicated. The incubation conditions for the preparation of MG-lysozyme are indicated in the figure. New peaks are labeled in bold.

a modification with Arg-pyrimidine, as this modification would result in a mass increase of 80 Da. All three of the newly detected peaks correspond to well-described modifications of proteins by methylglyoxal (22, 27). **Figure 5B** represents a spectrum of the molecular weight range between m/z 1170 and 1360. An additional peak was observed in the spectrum of MG-lysozyme at m/z 1256. The mass difference of 54 Da was very likely due to a modification of the ion at m/z 1202 with one methylimidazolone. In the spectrum of MG-lysozyme, produced upon incubation of lysozyme with 1 mM methylglyoxal for 30 h at room temperature, we were able to detect one methylimidazolone modification, as indicated by the appearance of a new peak at m/z 3196 (**Figure 5C**). Interestingly, in the spectrum of high-modified MG-lysozyme produced upon incubation of lysozyme with 25 mM methylglyoxal for 4 h at room temperature, the original ions at m/z 3126/3142 completely disappeared. Instead, two methylimidazolone modifications of the original ion at m/z 3142 were detected at m/z 3196 and 3250, as well as several other ions, which could not be identified at this time.

Finally, we compared native lysozyme to glycated lysozyme (AGE-lysozyme), produced upon incubation of lysozyme with 500 mM glucose for 3 weeks at 50 °C (**Figure 6**). **Figure 6A** represents the low molecular weight range from m/z 800 to 1360, in which three new molecular ions could be identified in the spectrum of AGE-lysozyme. The fragment at m/z 896 very likely represented a carboxymethylation of the N-terminal fragment at m/z 838, as the same m/z had already been observed in the model spectrum of CML-lysozyme (**Figure 2A**). The peak at

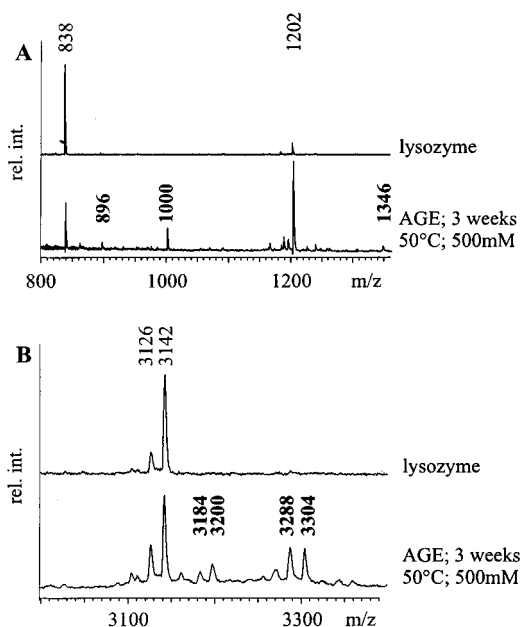


Figure 6. MALDI-TOF-MS peptide mapping analysis of AGE-lysozyme. In panel A, the molecular weight range between m/z 800 and 1370 is presented, and in panel B, the molecular weight region between m/z 3000 and 3500 is presented. m/z of identified ions is indicated. The incubation conditions for the preparation of AGE-lysozyme are indicated in the figure. New peaks are labeled in bold.

m/z 1000 was probably formed upon modification of the N-terminal fragment at m/z 838 with an Amadori product or glucosylamine, indicated by the mass increase of 162 Da. The third newly identified molecular ion was detected at m/z 1346. The same fragment had earlier been observed in the spectrum of 3-DG-lysozyme (**Figure 4A**). Therefore, it was very likely that this ion represented the imidazolone A-modified form of the peak at m/z 1202 (52). Several new peaks appeared in the spectrum of glycated lysozyme as compared to native lysozyme (**Figure 6B**). The molecular ions at m/z 3184 and 3200 could result from carboxymethylation of the fragments at m/z 3126 and 3142, because the same molecular masses were already observed in the spectrum of CML-lysozyme (**Figure 2B**). On the other hand, molecules with masses of m/z 3288 and 3304 represented the Amadori product or glucosylamine of the ions at m/z 3126 and 3142. Several other peaks were observed in the low and high molecular weight range and result from unidentified modifications.

DISCUSSION

Because the Maillard reaction has a major impact on the quality of processed and heated food products, the detection of protein glycation products in foods has always been a challenging area of investigation (1–20). Therefore, we employed MALDI-TOF-MS for this analytical problem. To date, there are few reports of the use of MS, such as MALDI-TOF-MS or ESI-MS, for the analysis of protein glycation (40–48). These publications established a mass increase of intact proteins, either isolated from *in vivo* sources or produced upon incubation of carrier proteins with reducing sugars (40, 41, 43–45, 47). Furthermore, two reports analyzed the primary glycation sites of the modified proteins (42, 48). To our knowledge, there is only one published report by Miyata et al. (46) dealing with the detection of specific AGE structures by MALDI-TOF-MS. The authors were able to detect a CML modification in the

N-terminal fragment of AGE-modified β_2 -microglobulin (AGE β_2 M), digested with endoproteinase Lys-C, after in vitro glycation or carboxymethylation of β_2 M.

The purpose of this investigation was to establish MALDI-TOF-MS peptide mapping for the determination of structurally specific Maillard products on glyated proteins. Resolution of MALDI-TOF-MS is not sufficient to determine low molecular weight modifications (e.g., 58 Da) on heterogeneously modified intact proteins (14.3 kDa). Therefore, we digested lysozyme with endoproteinase Glu-C, to obtain defined fragments of lower molecular weight (800–3500 Da). Thus, the relative mass increases due to a modification can be resolved on a molecular level. Furthermore, the following data were used to assign an observed mass shift to a structurally defined modification. In the protein data bank (53), the amino acid sequence and composition of lysozyme were obtained (SWISS-PROT: P00698). By theoretically digesting lysozyme in the protein data bank (53) with the endoproteinase Glu-C, we were able to link an observed peak to a defined peptide fragment or amino acid sequence. By comparing the information from the data bank with the well-described chemical mechanisms for the synthesis of our model substances, the combination of Δm measured and the assigned modification structure seems very plausible. Glu-C was used for digestion, because trypsin, which is commonly applied for peptide mapping, cleaves C-terminal to lysine and arginine. As these amino acids are modified by glycation, this enzyme is not the best choice.

In all of the synthesized protein modifications, we were clearly able to detect the introduced modification. In detail, CML-lysozyme and CEL-lysozyme showed modification of the N-terminal fragment (amino acids 1–7; **KVFGRC**E; m/z 838) as well as the following fragment in the amino acid sequence (amino acids 8–35; **LAAAMKRHGLDN**YRGYSLGNWV-**CAAKFE**; m/z 3142). The ion of m/z 3126 molecular mass, present in all mass spectra, was modified as well. The identity of this peptide, which seems to be a satellite peak of the peptide at m/z 3142, is not clear. It is not a fragment ion due to the loss of a small neutral molecule, such as H_2O or NH_3 , which is sometimes observed in MALDI-TOF-MS spectra (54). First, a mass difference of exactly 16 Da was determined, which cannot be explained by the loss of a neutral molecule. Furthermore, the technique of delayed extraction was applied, which suppresses postsource decay fragmentation. One reasonable explanation would be the presence of an isoform of lysozyme with a different primary amino acid sequence. The exchange of the amino acid tyrosine by phenylalanine or of serine by alanine would result in a 16 Da lighter peptide. Several isoforms of lysozyme are reported in the data bank for other species. Interestingly, in CEL-lysozyme, different degrees of modification, produced upon variation of the incubation procedure, could be detected. In these time course experiments, the relative intensity of the modified peaks clearly increased with the time of incubation and the amount of pyruvic acid used. In the spectrum of 3-DG-lysozyme, several new peaks appeared. Three of these ions could be assigned to imidazolone A-modified fragments due to their mass increase of 144 Da (52). In detail, fragment amino acids 8–35 (m/z 3142/3126) and fragment amino acids 120–129 (m/z 1202; **VQAWIRGCRL**) were modified.

In the spectrum of MG-lysozyme, we detected a modification in the N-terminal fragments (amino acids 1–7; m/z 838/amino acids 8–35; m/z 3142) as well as the C-terminal fragment (amino acids 120–129; m/z 1202) with a mass increase corresponding to methylimidazolone. Furthermore, we were able

to assign a CEL modification and a Arg-pyrimidine modification of the N-terminal fragment (amino acids 1–7; m/z 838). All of the detected modifications, methylimidazolone, Arg-pyrimidine, and CEL are well-described reaction products of the dicarbonyl compound methylglyoxal and proteins (22, 27). For MG-lysozyme, dependence of the modification degree on incubation conditions was shown. At a low concentration of methylglyoxal (1 mM), incubation of lysozyme resulted in the modification of the fragment at m/z 3142 (amino acids 8–35) with one methylimidazolone. In contrast, incubation with a higher concentration of methylglyoxal (25 mM) resulted in much more heterogeneous modifications of the same fragment. At least two methylimidazolone modifications were detected, as well as other unidentified reaction products.

Using the spectra of the model substances as references for modification products of glyated lysozyme (AGE-lysozyme), prepared upon incubation of lysozyme with D-glucose, we tried to identify specific structures of the Maillard reaction on the glyated protein. As expected, Amadori product/glucosylamine modifications, which cannot be distinguished by the methods applied, were observed. However, the presence of the Amadori product is more likely, because for MALDI-TOF-MS analysis the proteins were dissolved in acidic matrix solution, where glucosylamines are not stable (55). Additionally, we were able to detect specific modification products of the late stage Maillard reaction. The N-terminal fragment (amino acids 1–7; m/z 838) was carboxymethylated, as the peak detected at m/z 896 had already been observed in the CML-modified model protein. Furthermore, the C-terminal fragment (amino acids 120–129; m/z 1202) was likely modified with imidazolone A, as the same ion had already been observed in 3-DG-lysozyme. As incubation of proteins with reducing sugars such as D-glucose leads to protein glycation followed by sugar degradation and formation of 3-deoxyglucosone, the appearance of the ion at m/z 1346 in both the 3-DG-lysozyme and the glucose incubation seems very plausible. On the other hand, in the high molecular weight range between m/z 3000 and 3500, we were able to assign two peaks to CML modifications as well as modification with an Amadori product or glucosylamine on the ions at m/z 3126/3142. Again, these CML-modified peaks had already been observed in our model substance, CML-lysozyme. Importantly, in the range between m/z 3000 and 3500, several new peaks appeared in the spectrum of AGE-lysozyme as compared to native lysozyme. As advanced glycation of proteins with reducing sugars is a complicated process leading to the formation of a heterogeneous mixture of products, this was not unexpected and could be of particular interest for further investigations. Similar results were obtained after incubation of lysozyme with 500 mM D-glucose at 37 °C for 8 weeks. Even after incubation of lysozyme with 25 mM D-glucose at 50 °C for 3 weeks, resulting in the formation of a low-modified glyated lysozyme, modifications were still detectable (data not shown).

Tagami et al. (48) who studied structural modifications of lysozyme by the Maillard reaction found that after a 20 day incubation at 50 °C all of the 6 lysine and 10 of the 11 arginine residues were glyated. In contrast, our results indicated that the glyated protein is less intensively modified. A likely explanation is that Tagami et al. (48) performed glycation of the lyophilized protein at 75% relative humidity, whereas we incubated the protein and sugar in 10 mL of PBS at higher dilution.

These data demonstrate that MALDI-TOF-MS peptide mapping is a valuable technique for the detection of specific glycation products, formed during the Maillard reaction between

reducing sugars and proteins. In contrast, SDS-PAGE and MALDI-TOF-MS of intact proteins, techniques regularly used for mass determination of proteins, do not have enough resolving power to detect specific glycation products. However, the use of MALDI-TOF-MS for the absolute quantification of different glycation adducts is limited, because fragments of different molecular weight might be ionized and desorbed differently. This study also clearly confirmed the assumption that the major part of protein-bound Maillard products has not yet been identified and detected by the methods usually applied for analysis. MALDI-TOF-MS peptide mapping seems to be a promising tool for the identification of new structurally defined products of the late stage Maillard reaction.

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