JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Relative Quantification of N^{ϵ}-(Carboxymethyl)lysine, Imidazolone A, and the Amadori Product in Glycated Lysozyme by MALDI-TOF Mass Spectrometry

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The nonenzymatic glycation of proteins by reducing sugars, also known as the Maillard reaction, has received increasing recognition from nutritional science and medical research. In this study, we applied matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to perform relative and simultaneous quantification of the Amadori product, which is an early glycation product, and of N^{ϵ}-(carboxymethyl)lysine and imidazolone A, two important advanced glycation end products. Therefore, native lysozyme was incubated with D-glucose for increasing periods of time (1, 4, 8, and 16 weeks) in phosphate-buffered saline pH 7.8 at 50 °C. After enzymatic digestion with endoproteinase Glu-C, the N-terminal peptide fragment (*m*/*z* 838; amino acid sequence *K*VFG*R*CE) and the C-terminal peptide fragment (*m*/*z* 1202; amino acid sequence VQAWI*R*GC*R*L) were used for relative quantification of the three Maillard products. Amadori product, N^{ϵ}-(carboxymethyl)lysine, and imidazolone A were the main glycation products formed under these conditions. Their formation was dependent on glucose concentration and reaction time. The kinetics were similar to those obtained by competitive ELISA, an established method for quantification of N^{ϵ}-(carboxymethyl)lysine and imidazolone A. Inhibition experiments showed that coincubation with N^{α}-acetylargine suppressed formation of imidazolone A but not of the Amadori product or N^{ϵ}-(carboxymethyl)lysine. The presence of N^{α}-acetyllysine resulted in the inhibition of lysine modifications but in higher concentrations of imidazolone A. *o*-Phenylenediamine decreased the yield of the Amadori product and completely inhibited the formation of N^{ϵ}-(carboxymethyl)lysine and imidazolone A. MALDI-TOF-MS proved to be a new analytical tool for the simultaneous, relative quantification of specific products of the Maillard reaction. For the first time, kinetic data of defined products on specific sites of glycated protein could be measured. This characterizes MALDI-TOF-MS as a valuable method for monitoring the Maillard reaction in the course of food processing.

KEYWORDS: Maillard reaction; MALDI-TOF-MS; protein glycation; N^{ϵ}-(carboxymethyl)lysine; imidazolone A; Amadori product

INTRODUCTION

Proteins are extensively modified by nonenzymatic modifications in the course of food processing. Among these modifications, the Maillard reaction between the aldehyde group of reducing sugars and amino groups of proteins considerably influences various functional properties of foods (*1*, *2*). In the early stages of the Maillard reaction, the amino groups react with the aldehyde group of the reducing sugar, forming a Schiff base, which undergoes subsequent rearrangements to the Amadori product. Through oxidation, degradation, and rearrangement reactions, a heterogeneous group of advanced glycation end products (AGEs) is formed. In vivo, protein glycation and the formation of AGEs significantly affect the physical and physiological properties of long-lived proteins and thus contribute to diseases including diabetes mellitus or Alzheimer's (*3*−*6*). Despite intensive research, the identification and quantification of specific protein glycation products in vivo and in vitro is

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10.1021/jf020768y CCC: $25.00
Published on Web 12/07/2002

still a challenging analytical problem. To date, several techniques are applied for the detection and quantification of protein glycation products. Apart from chromatographic techniques including gas (*7*) and liquid chromatography (*8*), immmunochemical methods, mainly competitive ELISA (*9*), are used. Either of these techniques use secondary properties of particular Maillard products, such as fluorescence, UV-absorption, or affinity to highly specific antibodies, for identification and quantification. Thus, several structurally defined Maillard products have been identified or detected in vitro and in vivo over the last years (*10*−*15*).

Recently, mass spectrometric methods such as GC-MS, HPLC-MS with electrospray ionization, and MALDI-TOF-MS have been used for the analysis of protein glycation products (*16*−*18*). These techniques, generally applied for the detection of enzymatic protein modification, such as phosphorylation or glycosylation, are promising tools for monitoring Maillard products (*19*−*22*). For analysis of the molecular mass as an intrinsic molecular property, mass spectrometric techniques are highly sensitive. They furthermore allow the identification of the glycation sites, their relative reactivity, and the detection/distribution of different glycation products. We recently introduced MALDI-TOF-MS peptide mapping as a highly informative tool for the qualitative determination of early and advanced Maillard products (*23*, *24*). In this study, we used MALDI-TOF-MS to relatively quantify the Amadori product, an early glycation product, as well as the AGEs N^{ϵ}-(carboxymethyl)-lysine (CML) and imidazolone A. This technique allows analyses of the kinetics of the Maillard reaction for specific products and specific glycation sites.

MATERIALS AND METHODS

Chemicals. Lysozyme (chicken egg), D-glucose, sodium phosphate, α-cyano-4-hydroxycinnamic acid, dithiothreitol (DTT), bovine serum albumin (BSA), N^{α}-acetyl-L-lysine, N^{α}-acetyl-L-arginine, and *o*-phenylenediamine (OPD) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Endoproteinase Glu-C was purchased from Roche Diagnostics (Mannheim, Germany).

Glycation of Lysozyme. AGE-lysozyme was prepared as described previously (*25*). Briefly, lysozyme (0.7 mM) was dissolved with different amounts of D-glucose (500, 250, 100 mM) in 5 mL of phosphate buffered saline (PBS) (pH 7.8) and incubated for 1, 4, 8, and 16 weeks at 50 °C, followed by dialysis against doubly distilled water and lyophilization. The incubation mixtures were sterile filtered prior to incubation. At least three individual incubations/reaction condition were performed.

Inhibition of Protein Glycation. Lysozyme was incubated as described above, in the presence of competitors and inhibitors of the Maillard reaction as indicated. The N^{α}-acetyl-L-amino acids were added to achieve a molecular ratio of 3:1 for N^{α}-acetyl-L-amino acid- and lysozyme-bound ϵ-amino groups and guanidino groups, respectively. OPD was added equimolar to glucose. Briefly, lysozyme (0.7 mM) and D-glucose (500 mM) were incubated in 5 mL of phosphate-buffered saline (pH 7.8) for 2 weeks at 50 °C in the presence of N^{α}-acetyl-L-arginine (230 mM), N^{α}-acetyl-L-lysine (128 mM), OPD (500 mM), or OPD/N^{α}-acetyl-L-lysine (500 mM/43 mM).

Competitive ELISA Procedure. For immunoanalysis, the 96-well microtiter plates were coated with 0.5 μg/mL N^{ϵ}-(carboxymethyl)lysine-modified human serum albumin (CML-HSA) or imidazolone-HSA (*26*) in coating buffer (0.05 M carbonate buffer, pH 9.6, 200 μL) overnight at 4 °C. Unless otherwise noted, wells were washed twice with washing buffer after each step. Wells were blocked for 2 h with 3% skim milk in water. Then, 50 μL of competing antigen, standard (CML-HSA 0−3 μg/mL and imidazolone-HSA 0−10 μg/mL) in dilution buffer (0.05% Tween 20 and 0.2% BSA in 75 mM PBS), and 50 μL of anti-CML antibody (1:12 500 in dilution buffer) or anti-imidazolone antibody (1:1000 in dilution buffer) were added. The polyclonal anti-CML antibody (*27*) and the monoclonal anti-imidazolone antibody (*15*) had been prepared by us as described in the literature. Plates were incubated for 2 h and washed three times. Peroxidase conjugated anti-rabbit IgG for CML and anti-mouse IgG for imidazolone (diluted 1:5000 in 0.1% BSA in PBS) were added. After 1 h incubation, the wells were washed three times and color reaction was started with 150 μL/well tetramethylbenzidine diluted in substrate buffer. The reaction was stopped after 30 min by addition of 50 μL/well 2 N sulfuric acid, and absorption was measured at 450 nm. Results are given as % B/B_0, with B = absorbance in the presence of standard or sample and B_0 = absorbance in the absence of standard or sample. Samples were analyzed in triplicates. One Unit equals inhibition obtained with 1 μg/mL of CML-HSA or synthesized imidazolone-HSA (*9*).

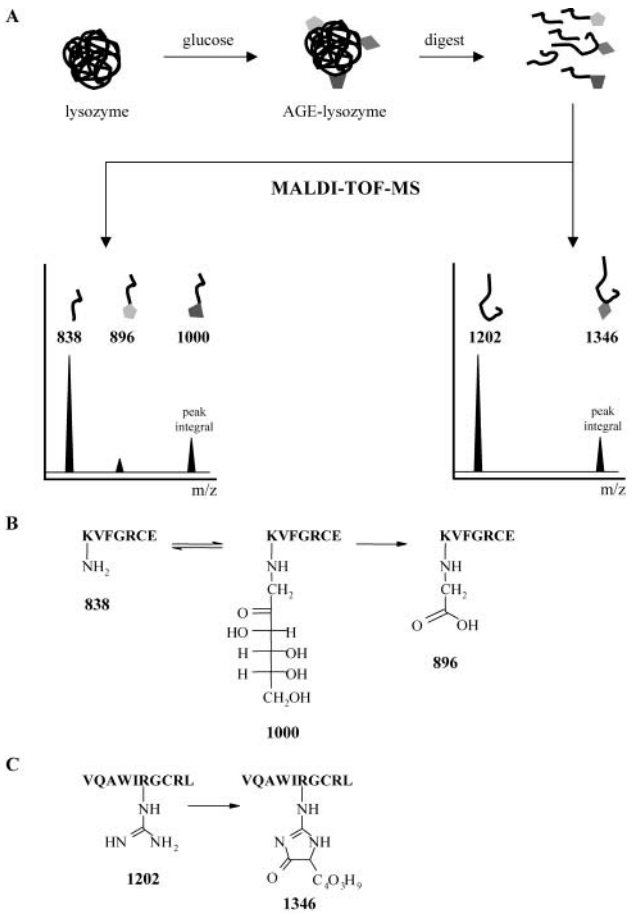
Endoproteinase Glu-C Digestions. Lysozyme and its glycation products were dissolved in 25 mM sodium phosphate buffer (pH 7.8) to a final concentration of 1 nmol/μL. For the digestion reaction, 5 μL of the lysozyme solutions, 3 μL endoproteinase Glu-C (3 μg), and 1 μL of 25 mM sodium phosphate buffer (pH 7.8) were incubated at 25 °C for 15 h. Prior to mass spectrometry, 1 μL of a 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). The protein from each incubation was digested in at least duplicate.

MALDI-TOF Mass Spectrometry. For MALDI-TOF-MS analysis, samples (500 pmol/μL) were diluted 1:50 in a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA with 33% acetonitrile. An aliquot (1 μL) 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 19 kV in the reflector mode. External calibration was performed by use of a standard peptide/protein mixture. Usually, each digest was spotted on at least three individual target positions and 100 individual spectra of each spot were averaged to produce a mass spectrum. Under these conditions, only single charged states were detected. The monitored mass range was *m*/*z* 600−2000.

RESULTS AND DISCUSSION

We attempted the simultaneous, relative quantification of N^{ϵ}-(carboxymethyl)lysine, imidazolone A, and the Amadori product on chicken egg lysozyme, glycated with increasing concentrations of D-glucose (AGE-lysozyme) for various periods of time. The AGE-lysozyme was digested with endoproteinase Glu-C and MALDI-TOF mass spectra were recorded. A comparison of the peptide fragment patterns obtained with those derived from a theoretical digest in a protein data bank (e.g. http://www.expasy.com) provides information concerning the preferential glycation site of the protein. By linkage of these data with the amino acid sequence of a particular fragment, the glycation site could be defined on the molecular level. On the basis of our recent analysis (*23*), we used two identified fragments, the N-terminal fragment (amino acid 1−7; sequence *K*VFG*R*CE; *m*/*z* 838) and the C-terminal fragment (amino acid 120−129; sequence VQAWI*R*GC*R*L; *m*/*z* 1202), for the relative quantification of the three glycation products **(Scheme 1A).** The region and *m*/*z* range of these fragments allow isotope specific resolution and comparison of the integrals of the monoisotopic peaks. Furthermore, the N-terminal fragment contains one lysine and one arginine residue, whereas the C-terminal fragment contains two arginine residues but no lysine. Thus, it was possible to distinguish clearly between arginine and lysine modifications. To validate the reliability of this assay, several incubations of AGE-lysozyme were prepared for each reaction condition. Each AGE-lysozyme was digested independently at

Scheme 1. Relative Quantification Using MALDI-TOF-MS[a]



[a] (A) Lysozyme was incubated with different concentrations of D-glucose for various times, and the modified protein was digested with endoproteinase Glu-C resulting in the formation of defined peptide fragments. The digest was subject to MALDI-TOF-MS. The monoisotopic peak integral of the modified peptides (*m*/*z* 896, 1000, and 1346) was measured in comparison to the monoisotopic peak integral of the unmodified peptides (*m*/*z* 838 and 1202), defined as 1.0, producing the relative integrals. The amino acid sequence, the molecular mass of the N-terminal (B) and the C-terminal (C) peptides, and the structure of the quantified glycation products are presented.

least in duplicate and spotted on at least three different positions of the target used for MALDI-TOF mass spectrometry.

MALDI-TOF Mass Spectrometry of AGE-Lysozyme Digests. Figure 1 depicts the MALDI-TOF mass spectra of lysozyme incubated with 500 mM D-glucose for various periods of time and digested with endoproteinase Glu-C. While the N-terminal fragment is represented at *m*/*z* 838 (amino acid sequence: *K*VFG*R*CE), the C-terminal fragment is identified at *m*/*z* 1202 (amino acid sequence: VQAWI*R*GC*R*L). In the absence of D-glucose, only the fragments *m*/*z* 838 and 1202 were detectable in this mass region (**Figure 1A**). Panels B−E show the lysozyme sample glycated for 1, 4, 8, and 16 weeks, respectively. Incubation of lysozyme with D-glucose resulted in the appearance of new peaks that were absent from the spectra of native lysozyme, representing glycation products of the two peptide fragments analyzed (*m*/*z* 838 and 1202). As described recently (*23*), the additional ions at *m*/*z* 896 and 1000 were assigned to a N^{ϵ}-(carboxymethyl)lysine and an Amadori product modification of the N-terminal fragment, consistent with the mass increase of 58 and 162 Da, respectively (**Scheme 1B**). The new ion appearing in the right-hand panel at *m*/*z* 1346 was assigned to imidazolone A, a modification product of an arginine residue in the C-terminal fragment, due to the mass increase of

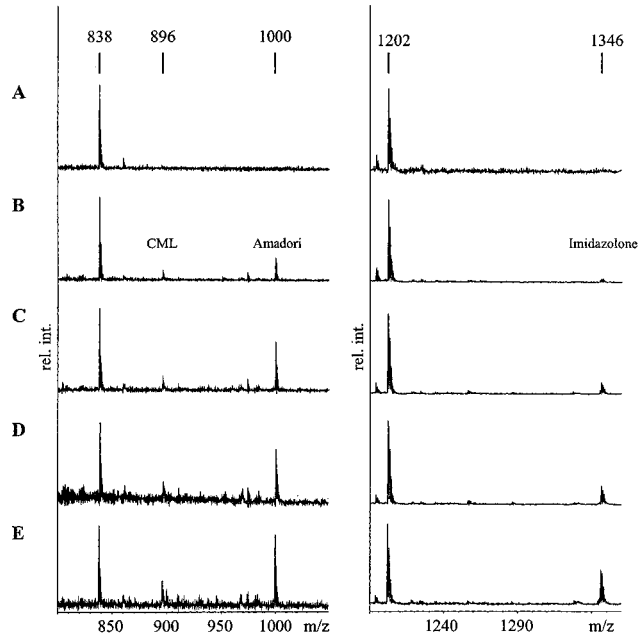


Figure 1. MALDI-TOF mass spectra of digested lysozyme incubated with 500 mM D-glucose. Lysozyme was incubated with 500 mM D-glucose and incubated for various periods of time: (A) native lysozyme; (B) 1 week; (C) 4 weeks; (D) 8 weeks; (E) 16 weeks. Spectra for the N-terminal peptide fragment are given on the left panel, and spectra for the C-terminal peptide fragment are presented on the right panel. Values for *m*/*z* of quantified peaks were as indicated.

144 Da (**Scheme 1C**) (*23*). The relative intensity of spectra representing the modified peptides increased with incubation time, as compared to those of the native peptide. Similar results were observed for lysozyme incubated with 100 and 250 mM D-glucose (data not shown).

For relative quantification, the peak integral representing the peak area of the monoisotopic peak was measured. The peak integral of the unmodified N- and C-terminal peptides, appearing at *m*/*z* 838 and 1202, respectively, was defined as 1.0 (standard). The relative integral of the product peaks at *m*/*z* 896 (CML-modified peptide), *m*/*z* 1000 (Amadori-modified peptide), and *m*/*z* 1346 (imidazolone A-modified peptide), respectively, was then calculated on the basis of the internal standards at *m*/*z* 838 and 1202. Besides the two native peptides and the major glycation products, in some spectra two minor ions were observed at *m*/*z* 861 and 1225, representing $[M + Na]^+$, and also some minor peaks which probably derive from glycation products which have not yet been identified. These peaks were negligible for quantification.

Relative quantification of the products, depending on sugar concentration and reaction time, is given in **Figure 2** (Amadori product, CML) and **Figure 3** (imidazolone A).

Relative Quantification of Amadori Product and N^{ϵ}-(Carboxymethyl)lysine. Integrals of the modified peptides at *m*/*z* 896 and 1000 were standardized to the peak integral of the unmodified peptide at *m*/*z* 838. Formation of the Amadori product was dependent on both sugar concentration and incubation time (**Figure 2A**). Incubations for 8 and 16 weeks with 250 or 500 mM D-glucose showed a relatively large standard deviation, which is most likely due to excessive protein glycation resulting in incomplete digestion of the modified protein (*28*, *29*). In contrast shorter incubation time or lower concentrations of D-glucose (100 mM) yielded smaller standard deviations. **Figure 2B** presents the relative integral percentage of the

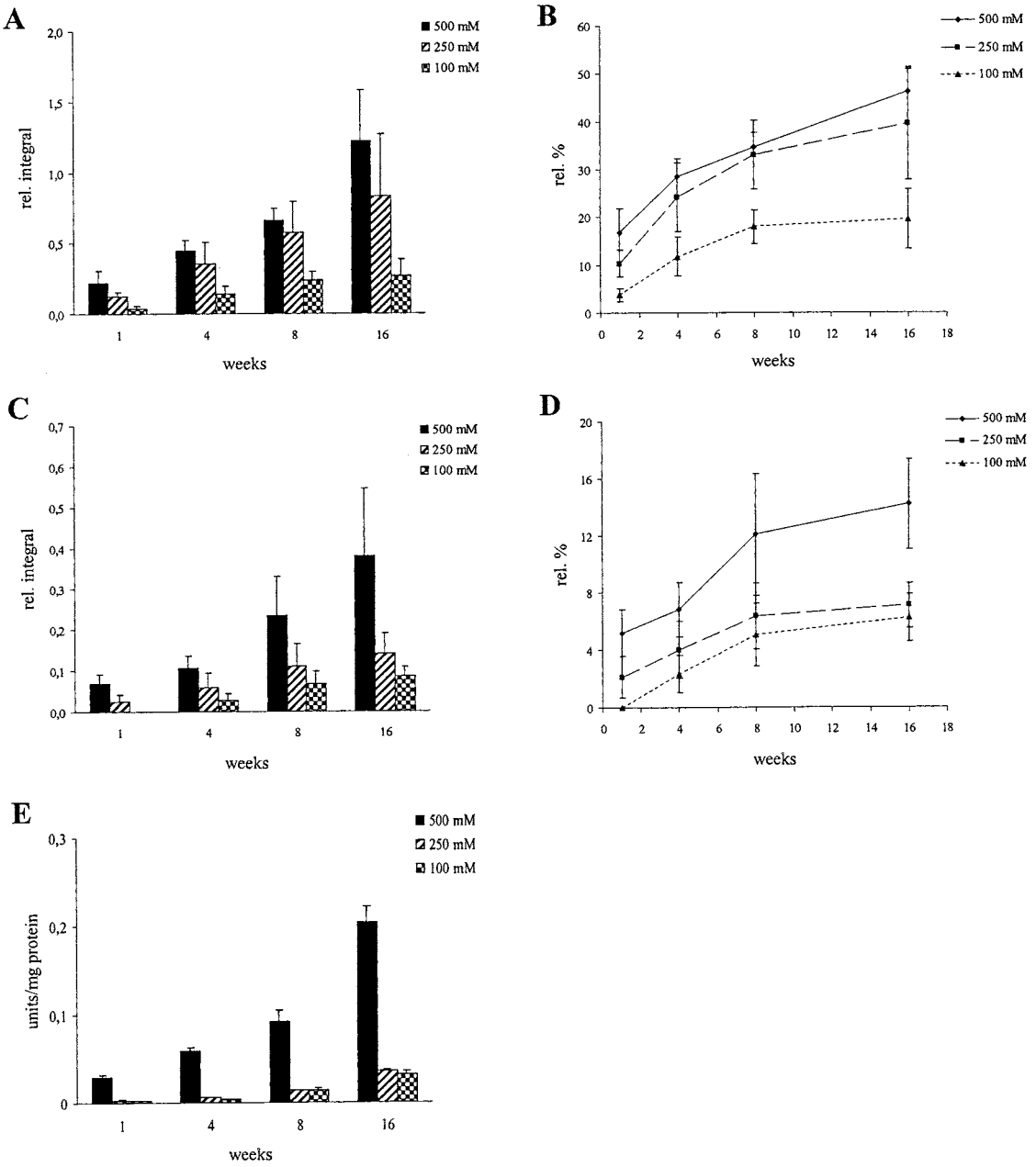


Figure 2. Relative amounts of N^{ϵ}-(carboxymethyl)lysine and the Amadori product on the N-terminal peptide fragment: (A) relative quantification of the Amadori product produced upon incubation of lysozyme with the indicated concentrations of D-glucose for various time; (B) change of the relative integral percentage of the Amadori product on the N-terminal fragment of lysozyme plotted against the time of incubation; (C) relative quantification of CML produced upon incubation of lysozyme with the indicated concentrations of D-glucose for various time points; (D) change of the relative integral percentage of CML on the N-terminal fragment of lysozyme plotted against the time of incubation; (E) formation of glycation end products determined by a competitive ELISA for CML. Data are presented as mean ± standard deviation (A–D, $n = 10$; E, $n = 3$).

Amadori product of the N-terminal fragment (*m*/*z* 838) depending on sugar concentrations. Relative integral percentage was obtained by dividing the integral of Amadori product (P^{P}_{1000}) by the sum of all product (P^{P}) and educt ($P^{E}_{838} = 1$) integrals:

$$P^{P}_{1000}/(P^{P}_{1000} + P^{E}_{838} + P^{P}_{896})$$

With this approach it became possible to obtain quickly kinetic data for the Maillard reaction of this protein site. However MALDI-TOF-MS did not permit interspectral quantitative comparison of different ion species, as desorption and ionization of different ion species may be completely different. Yet, an intraspectral comparison could reliably be performed. In time course studies an identical fragment species was quantified relative to the same standard. This implies that the conditions of ionization and desorption are the same for each time point analyzed. In the presence of 100 mM D-glucose, the concentration of the Amadori product continuously increased during the first 8 weeks of incubation, reaching a plateau thereafter **(Figure 2B)**. With 250 and 500 mM D-glucose, equilibrium was not fully reached after 16 weeks. These kinetics of Amadori product formation are consistent with the literature (*30*, *31*).

Next, we relatively quantified the advanced glycation end product N^{ϵ}-(carboxymethyl)lysine on the N-terminal fragment (**Figure 2C,D**). As shown in **Figure 2C**, an increase in CML-modified peptide formation was observed with increasing sugar

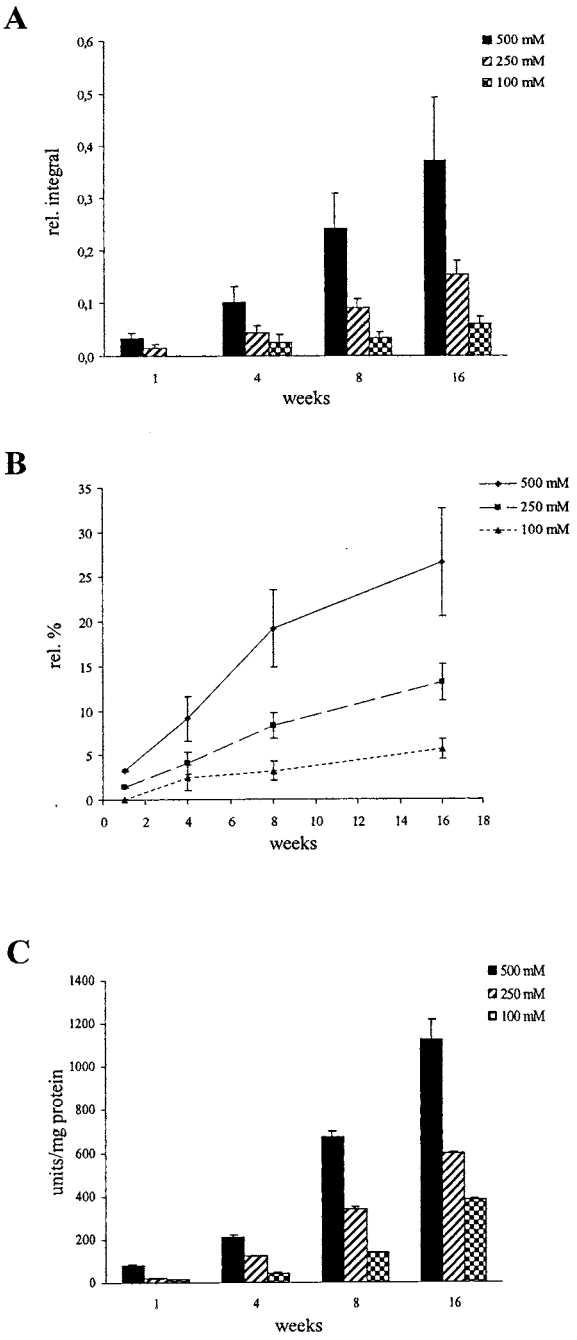


Figure 3. Relative amounts of imidazolone A on the C-terminal peptide fragment: (A) relative quantification of imidazolone A produced upon incubation of lysozyme with the indicated concentrations of D-glucose for various time; (B) change of the relative integral percentage of imidazolone A on the N-terminal fragment of lysozyme incubated with different concentrations of D-glucose plotted against the time of incubation; (C) results obtained from a competitive ELISA for imidazolone A. The data are presented as mean ± standard deviation (A, B, $n = 10$; C, $n = 3$).

concentration and incubation time. Formation of CML could not be detected after 1 week of incubation with 100 mM D-glucose. In contrast, we were able to detect a weak signal for an Amadori product modified peptide under these conditions. This is consistent with the formation of CML by oxidative cleavage of the Amadori product or by interaction of proteins with glyoxal or other sugar degradation products (*10*, *32*). Thus, formation of the Amadori product should precede accumulation of CML. Indeed, CML was detected after incubation of lysozyme with 100 mM D-glucose for 4 weeks. **Figure 2D** depicts the increase in relative integral percentage for CML with increasing incubation times and sugar concentrations. While a constant increase of the kinetic curve would have been expected for the advanced glycation end product CML, the flattening observed may be due to inefficient proteolysis of the modified N-terminal or poor desorbtion/ionization of the peptide compared to the C-terminal peptide.

Relative Quantification of Imidazolone A. Imidazolone A is an advanced glycation end product formed upon reaction of the sugar degradation product 3-deoxyglucosone with an arginine side chain. Following the protocol applied to the determination of Amadori product and CML, imidazolone A was subjected to relative quantification (**Figure 3A**). The relative integral of the modified peptides at *m*/*z* 1346 was standardized to the peak integral of the unmodified C-terminal peptide fragment (*m*/*z* 1202) of lysozyme. The imidazolone A signal increased with D-glucose concentration and incubation time. For any glucose concentration, the imidazolone A signal roughly doubled from each time point to the next. Likewise, doubling the glucose concentration also led to a corresponding increase in imidazolone A. **Figure 3B** shows the kinetics of imidazolone A formation where the relative integral was plotted against the incubation time at three different concentrations of D-glucose (100, 250, and 500 mM). Again, a clear increase of imidazolone A was observed with increasing time and sugar concentration. In contrast to CML (**Figure 2D**), the kinetic curve observed for imidazolone A derived from the 100 and 250 mM glucose incubations was almost linear, as theoretically expected for an advanced glycation end product. In the presence of 500 mM glucose, the curve of imidazolone A formation flattens after the 8 weeks of incubation. This might be due to incomplete digestion of the protein after excessive glycation, resulting in protein cross-linking. The same phenomenon was also observed after 24 weeks of incubation at all sugar concentrations (data not shown).

Inhibition of Protein Glycation. Identification of glycation products in MALDI-TOF-MS spectra is achieved by comparing the mass increases observed with (i) theoretically expected data and (ii) synthetic model peptides (*23*). To confirm that the adduct peaks detected originate from glycation reaction, incubations were also performed in the presence of glycation inhibitors (*33*). **Figure 4A** shows the spectra of native lysozyme digested with endoproteinase Glu-C for the molecular weight ranges between *m*/*z* 800−1050 (left panel) and *m*/*z* 1190−1360 (right panel). The spectrum of lysozyme incubated with 500 mM D-glucose for 2 weeks at 50 °C (**Figure 4B**) is characterized by the appearance of two new signals at *m*/*z* 896 and 1000, representing a CML modification and a modification with the Amadori product, respectively (left panel), while a signal corresponding to an imidazolone A modification was detected at *m*/*z* 1346 (right panel).

Next, lysozyme was incubated with 500 mM D-glucose (2 weeks, 50 °C) in the presence of N^{α}-acetyl-L-arginine, a competitor for arginine modifications in the course of the Maillard reaction (**Figure 4C**). Indeed the signal for imidazolone A, a modification formed upon reaction of 3-deoxyglucosone with an arginine side chain, completely disappeared (right panel). In contrast, only a weak suppression of CML and the Amadori product could be observed (left panel). This may be a tribute to the reaction of D-glucose with N^{α}-acetyl-L-arginine, resulting in a lower sugar concentration. N^{α}-acetyl-L-lysine served as a competitor for primary amino groups in the course of the Maillard reaction (**Figure 4D**). As expected, this resulted in

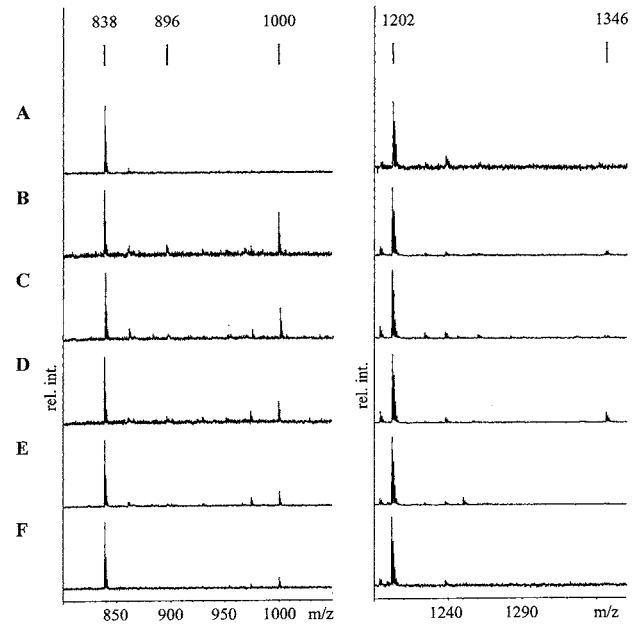


Figure 4. MALDI-TOF mass spectra of digested lysozyme incubated with 500 mM D-glucose and several competitors. Lysozyme was incubated with 500 mM D-glucose for 2 weeks at 50 °C in the presence of several competitors of the protein glycation: (A) native lysozyme; (B) 500 mM D-glucose; (C) 500 mM D-glucose/230 mM N^{α}-acetyl-L-arginine; (D) 500 mM D-glucose/125 mM N^{α}-acetyl-L-lysine; (E) 500 mM D-glucose/500 mM *o*-phenylenediamine; (F) 500 mM D-glucose/500 mM *o*-phenylenediamine/45 mM N^{α}-acetyl-L-lysine. On the left-hand panel, the spectra for the N-terminal peptide fragment and, on the right-hand panel, the spectra for the C-terminal peptide fragment are presented. Values for *m*/*z* of quantified peaks were as indicated.

the suppression of the CML modification (*m*/*z* 896) and the Amadori product (*m*/*z* 1000), while an increase in the signal representing the imidazolone A modification could be observed. Amines, such as N^{α}-acetyl-L-lysine, catalyze sugar degradation reactions, resulting in an increasing formation of dicarbonyl compounds such as 3-deoxyglucosone (*34*). Furthermore, incubation was performed in the presence of *o*-phenylenediamine (OPD), a well-characterized inhibitor of the Maillard reaction blocking reactive α-dicarbonyl compounds, such as 3-deoxyglucosone. (*33*). On the left-hand panel, a significant suppression of the Amadori product and a disappearance of the CML modification were observed. This is most likely due to an overall decrease in the concentration of accessible sugar, resulting in a lower concentration of the primary glycation intermediate, the Amadori product. On the other hand, CML can be formed by two alternative glycation pathways, involving (i) glycoxidation of the initially formed Amadori product (*10*) or (ii) the reaction of sugar degradation products (e.g. glyoxal) with amino groups (*32*). As dicarbonyls such as glyoxal are bound by OPD, disappearance of CML is consistent with this mechanism. Complete disappearance of the assigned imidazolone A peak was observed (**Figure 4E**), which can be explained by the reaction of OPD with 3-deoxyglucosone. As shown in **Figure 4F**, inhibition was even enhanced when a combination of N^{α}-acetyl-L-lysine and OPD was added to the incubation mixture. The two advanced glycation end products, CML and imidazolone A, completely disappeared while the signal intensity of the Amadori product modified peptide decreased even more, as observed for OPD alone. These findings support the recently postulated structures (*23*, *24*).

Comparison of MALDI-TOF-MS and ELISA. Finally, we compared the relative quantification of CML and imidazolone A obtained by MALDI-TOF-MS with that by a competitive ELISA, an established technique for the quantification of AGEs (**Figures 2** and **3**). By comparison of data presented in **Figure 2C** with those of **Figure 2E**, it becomes evident that relative quantification of CML and imidazolone A with MALDI-TOF-MS corresponded with the results obtained by competitive ELISA (**Figure 3A,C**).

Competitive ELISA can be used—after proper validation—for absolute quantification. On the other hand, MALDI-TOF-MS enables absolute quantification only, when standards are available which are labeled with stable isotopes, because differences in desorption and ionization rate of different modifications cannot be excluded. Still MALDI-TOF-MS combined with peptide mapping as applied here can be used for relative quantification and offers the advantage that kinetic data for different glycation sites can be obtained. The MALDI-TOF-MS protocol also offers the advantage of a simultaneous assessment of different products of the nonenzymatic protein glycation. Immunochemical techniques, in contrast, are restricted to quantification of only one epitope/assay. Furthermore, development of a new immunoassay is very time-consuming and laborious. The parallel detection of several AGEs combined with the high-throughput potential of MALDI-TOF-MS should allow this technique to become a valuable analytical tool for researchers working with nonenzymatic protein glycation.

In this study, protein glycation in aqueous solution was investigated. Therefore, a relatively low reaction temperature was chosen to avoid protein precipitation. However, the method can also be applied to proteins glycated at higher temperatures for example as present after food processing.

In summary, it was shown that MALDI-TOF-MS in combination with endoproteinase Glu-C digestion serves as a valuable analytical tool for the parallel, relative detection and quantification of the Amadori product, an early stage Maillard product, as well as N^{ϵ}-(carboxymethyl)lysine and imidazolone A, two important advanced glycation end products. Furthermore it was for the first time possible to obtain kinetic data for different defined products on specific sites of glycated proteins. Therefore, this new technique should be of particular interest for further investigations in the field of food analysis by monitoring the Maillard reaction during the process of storing and preparation.

ABBREVIATIONS USED

AGE, advanced glycation end product; BSA, bovine serum albumin; CML, N^{ϵ}-(carboxymethyl)lysine; 3-DG, 3-deoxyglucosone; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbend assay; HSA, human serum albumin; GC-MS, gas chromatography−mass spectrometry; HPLC-MS, high-performance liquid chromatography−mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; OPD, *o*-phenylenediamine; PBS, phosphate-buffered saline.

ACKNOWLEDGMENT

This study was supported by the “Fond der chemischen Industrie”. We thank Bruker Daltonik GmbH (Bremen, Germany) for providing the Biflex III MALDI-TOF mass spectrometer. We are grateful to Dr. Markus Kostrzewa, Bruker Saxonia Analytik GmbH (Leipzig, Germany), for helpful discussions.

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Received for review July 15, 2002. Revised manuscript received September 30, 2002. Accepted October 2, 2002.

JF020768Y