Monitoring Glycation of Lysozyme by Electrospray Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry (ESI-MS) was used to study the glycation of lysozyme by D-glucose (LZM-G) and by D-fructose (LZM-F) under dry heating conditions in the presence and in the absence of oxygen. ESI-MS proved to be a precise method for monitoring protein glycation with respect to following the extent of glycation and changes in the glycoconjugate profile with time. The ESI-MS spectrum of glycated LZM revealed a heterogeneous distribution of glycoforms of LZM at different reaction stages. D-Glucose showed a higher level of reactivity with the amino groups of LZM than D-fructose, both in the presence and in the absence of oxygen. The presence of oxygen in the reaction system induced oxidative side reactions, which competed with and slowed the initial rate of formation of Amadori or Heyns products. The more reactive glycoxidation products formed during the initial stages of incubation in the presence of oxygen accelerated the rate of glycation during the later stages of incubation and increased the involvement of arginine residues of LZM in the glycation reaction. The interaction between the initial glycoxidation product(s) of the reducing sugars and intact lysozyme during the later stages of incubation was observed by the appearance of a different cluster of glycoconjugates in the mass spectrum during the latter stages of incubation. The molecular weight differences between the molecular ions of the new cluster of glycoconjugates are consistent with the formation of D-glucosone from the autoxidation of D-glucose or from the oxidative cleavage of the glucose−lysozyme imine adduct in the lysozyme−glucose system. The effect of oxygen-induced glycoxidation on the glycation reaction was also more pronounced in the LZM-G system compared with that in the LZM-F system.

Keywords: *Glycation; oxidation; lysozyme; D-glucose; D-fructose; D-glucosone; dehydration; electrospray mass spectrometry; reactivity*

INTRODUCTION

The importance of the Maillard reaction involving proteins and reducing sugars in vitro (in food systems) and in vivo (in biological systems) cannot be overstated. Protein glycation reactions, and the subsequent modification of protein functionality in food systems, as well as the physiological and pathological consequences of protein glycation in vivo have been the subject of much investigation in recent years.

Protein glycation reactions may be considered as those that occur during the early and intermediate stages of the Maillard reaction involving proteins and reducing saccharides and in which the protein is still recognizable as a glycoconjugate or as a cross-linked polymer of the original protein. These reactions include the initial condensation reaction to form Amadori or Heyns intermediate rearrangement products, dehydration of the sugar moieties of intact Amadori or Heyns products, and inter- and intramolecular cross-linking reactions to form soluble polymers.

The effect of glycation on food proteins may be beneficial or detrimental, depending on the extent of glycation. Controlled, limited glycation has been reported to impart beneficial effects to proteins. Some of the beneficial effects of food protein glycation include improved emulsifying properties (Nakamura et al., 1992b; Shu et al., 1996; Saeki, 1997; Handa and Kuroda, 1999), improved gelation and water holding capacity (Mat Easa et al., 1996), and improved foaming and solubility properties (Mat Easa et al., 1996; Kato et al., 1990, 1991, 1992; Nakamura et al., 1991, 1992a,b). Extensive glycation, on the other hand, results in protein cross-linking and loss of protein solubility (Yeboah et al., 1999). Glycation is also associated with the loss of the nutritional value of food proteins, due to the unavailability of blocked lysine residues (Naranjo et al., 1998) and damage to other essential amino acids such as tryptophan (Moreaux and Birlouez-Aragon, 1997), as well as the loss of protein digestibility (Hurrel, 1990; Umetsu and Van Chuyen, 1998). Extensive glycation of proteins has also been implicated in the formation of toxic advanced glycation end products (AGEs) (Koschinsky et al., 1997). Other studies have, however, reported some beneficial effects associated with advanced Maillard reaction of proteins. These include the formation of compounds with antioxidant (Hayase et al., 1990; Chuyen et al., 1998), anticarcinogenic, and antimutagenic properties (Aeschbacher, 1990).

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The use of functionally improved glycated proteins in food systems is more desirable when compared with chemically modified food proteins, as it does not pose the problem of residual chemicals in the food system (Kato et al., 1996). However, the application of heat-induced glycated food proteins in the food system as functional ingredients will require precise monitoring of the processing and storage controls and the use of evaluation methods that provide detailed information at all stages of handling to ensure product quality and consistency.

Protein glycation in food systems has been followed by various methods, including the measurement of browning at A_{420nm} (Labuza and Saltmarch, 1981; Davies et al., 1998), Maillard fluorescence at 340−350 nm excitation and 400−440 nm emission (Morales et al., 1996; Morals and van Boekel, 1997; Suarez et al., 1995), and reacted or blocked lysine residues by the furosine assay (Finot and Magnenat, 1981; Moreaux and Birlouez-Aragon, 1997; Kubow et al., 1993) and by the fluorescamine assay (Yeboah et al., 1999; Yaylayan et al., 1992). These methods of evaluation, particularly the browning and fluorescence measurements, evaluate the bulk changes that occur during the Maillard reaction and are therefore not adequate for precise monitoring of protein glycation.

Mass spectrometry has been increasingly used to evaluate and characterize protein glycation, both in food and in biological systems. Soft ionization mass spectrometric techniques, such as electrospray ionization (ESI-MS), and matrix-assisted laser-induced ionization time-of-flight mass spectrometry (MALDI-TOF) provide accurate relative molecular mass values (M_r) of labile biomolecules such as proteins in the order of 0.01% at 20 kDa. Protein modifications, such as the formation of Amadori products, dehydration, oxidation, and cross-linking, that occur during protein glycation can therefore be monitored by soft ionization mass spectrometry. ESI-MS has been used to study lactosylation of β-lactoglobulin in milk during processing and to locate the glycation sites of β-lactoglobulin (Leonil et al., 1997; Morgan et al., 1997) and to study the association behavior of glycated proteins (Morgan et al., 1999). ESI-MS has also been used to study the glycation of hemoglobin in vivo (Al-Abed et al., 1999). Others have used collision-induced tandem mass spectrometry to study the fragmentation pattern of glycated peptides (Molle et al., 1998; Zigrovic et al., 1998). More recently, ion-trap mass spectrometry has been used to study the glycation of lysozyme (Tagami et al., 2000).

In this study, we report the use of ESI-MS for evaluating protein glycation, the identification of glycated species of lysozyme formed during the glycation of lysozyme with D-glucose and D-fructose, and the effect of oxygen on the distribution profile of the glycated species of lysozyme.

MATERIALS AND METHODS

Materials. Chicken egg white lysozyme (LZM) (>96% purity) and D-glucose and D-fructose were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade from Fisher Scientific (Nepean, ON, Canada). Ultrapure water from a Nanopure water purification system (Barnstead, Thermolyne, Dubuque, IA) was used throughout the study.

Sample Preparation. Lysozyme (25 g) and D-glucose (LZM-G) or D-fructose (2.25 g) (LZM-F) were dissolved in water (50 mL) to give a protein to sugar molar ratio of 1:6 (approximate 1:1 ratio of sugar carbonyl to lysine residues). The solutions were adjusted to pH 7 with dilute HCl or NaOH, freeze-dried, and stored at −20 °C until further analysis. Quantities of the freeze-dried protein−sugar mixtures (1 g) were placed in 10 mL glass vials, covered with perforated aluminum foil, and incubated at 50 °C in sealed glass desiccators maintained at a relative humidity of ∼65% (Greenspan, 1977) with saturated aqueous KI solution throughout the incubation period. The samples were incubated for 1, 2, 5, 10, and 14 days in separate desiccators to prevent repeated opening and closing of the sealed desiccators. After incubation, the samples were stored at −20 °C until they were analyzed. Two separate experiments were conducted, one in the presence of air (oxygen) and the other in the absence of air (in nitrogen). The water used to prepare the saturated aqueous KI solution for the incubations done in nitrogen was previously degassed and saturated with ultrapure nitrogen.

Fluorescamine Assay. A modification of the fluorescamine assay (Yaylayan et al., 1992) was used to determine the proportion of reacted ϵ-amino groups of lysozyme in the incubated samples. A quantity (25 mg) of the incubated samples was suspended in Nanopure water (10 mL), vortexed, and sonicated for 30 min. The resulting solution was filtered through 0.22 μm syringe filters. The filtrate was diluted (10 times), and a 200 μL aliquot of the diluted solution was placed in a centrifuge tube containing 4 mL of borate buffer (0.2 M potassium borate, pH 8.5). An aliquot (1 mL) of fluorescamine reagent (15 mg in 100 mL of acetone) was added rapidly while vortexing. Borate buffer (4.2 mL) containing no protein was used as a blank. Fluorescence was measured on a Kontron spectrofluorometer (Kontron Instruments SFM 25 spectrofluorometer, Zurich, Switzerland) at excitation and emission wavelengths of 390 and 475 nm, respectively. The fluorescence results were normalized by the protein content of the respective solutions. The protein content of the diluted filtrate (above) was determined according to the Lowry method (Lowry et al., 1951). All assays were done in triplicate.

Reversed Phase HPLC. The incubated samples were subjected to RP-HPLC to separate lysozyme (monomeric glycated and nonglycated) from free sugars and other reaction products. Quantities of the incubated samples (5 mg) were suspended in 0.1% aqueous trifluoroacetic acid (TFA) (1 mL), vortexed, and sonicated for 15 min. The resulting solution was filtered through 0.22 μm syringe filters, and 50 μL of the filtrate was injected for HPLC analysis. The HPLC analysis was performed on a Beckman Gold HPLC system equipped with a UV diode array detector (Beckman Instruments, Inc., Fullerton, CA) and a Shimadzu spectrofluorometer (RF-551, Shimadzu Corp., Kyoto, Japan). The UV detector was set at 214 nm, and the fluorometer was set to measure Maillard fluorescence at 350 and 440 nm for excitation and emission, respectively. Separation was achieved on a reversed phase analytical column (Vydac C18 column, 218TP54; The Separation Group, Mojave, Hesperia, CA), fitted with a guard column (Brownlee 3 cm C18; Chromatographic Specialties Inc., Brockville, ON, Canada). A gradient mobile phase system [solvent A (0.1% TFA in Nanopure water) and solvent B (0.1% TFA in acetonitrile)], with a flow rate of 1 mL/min and a linear gradient of 0% B to 50% B in 60 min was used for elution. Fractions were collected, freeze-dried, and subjected to electrospray mass spectrometry.

Mass Spectrometry. The freeze-dried HPLC fractions were reconstituted in 70% aqueous acetic acid and analyzed by electrospray mass spectrometry using a triple-quadrupole mass spectrometer (API III MS/MS system, SCIEX, Thornhill, ON, Canada). Samples were infused into the electrospray ion source (fused silica capillary of 100 μm i.d.) at a rate of 1 μL/min from a low-pressure infusion pump (model 22, Harvard Apparatus, South Natick, MA).

Determination of Molecular Mass and Average Sugar Loading Values (SLV) of LZM. The molecular masses of LZM and its glycated species (LZM-G and LZM-F) in the incubation mixtures were determined as an average of three determinations from three different multiply charged molecular ion states (+7, +8, and +9). The relative proportions of

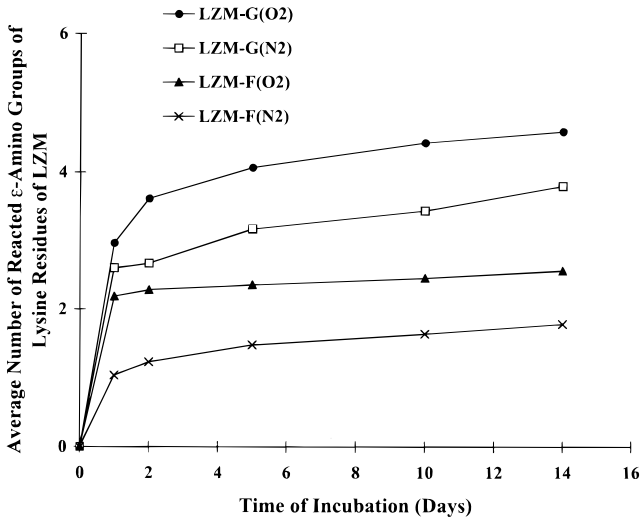


Figure 1. Average number of ϵ-amino groups of lysine residues of lysozyme that had been glycated over the 14 day incubation period as determined by the fluorescamine assay.

glycated and unglycated species of LZM identified in the incubated samples were estimated from the relative intensities of their corresponding molecular ions in the mass spectrum, as a ratio of the total intensity of all identified molecular ion peaks. The relative proportions of each identified glycoform were also determined at three different multiply charged states (+7, +8, and +9) and averaged. The average sugar load per molecule of glycated lysozyme (SLV), with time of incubation, was determined as a weighted average of the identified glycoforms of lysozyme.

RESULTS

Fluorescamine Assay. The progress of the reaction between the ϵ-amino groups of LZM and the carbonyl groups of D-glucose or D-fructose is shown in Figure 1. The results show that the initial rate of glycation of the ϵ-amino groups of LZM by D-glucose is higher than that by D-fructose, under both conditions of incubation (incubation in the presence and in the absence of oxygen). Both D-glucose and D-fructose showed a higher initial rate of glycation in the presence of oxygen than in its absence. By the first day of incubation, D-glucose had reacted with an average of 2.9 and 2.6 ϵ-amino groups of LZM, while D-fructose had reacted with an average of 2.2 and 1 ϵ-amino groups in the presence and absence of oxygen, respectively.

Estimation of the Reactivity of D-Glucose and D-Fructose with Lysozyme by ESI-MS. The rate of glycation of LZM was also determined as the average SLV per molecule of LZM at the different stages of incubation by ESI-MS. A plot of the SLV of LZM versus time of incubation is shown in Figure 2. The results show that, similar to the fluorescamine assay, the initial rate of glycation of lysozyme by D-glucose was higher than that by D-fructose in both the presence and the absence of oxygen. Unlike the fluorescamine assay, the results of the SLV show that the initial rate of glycation of LZM by D-glucose was higher in the absence of oxygen than in its presence. In the presence of oxygen, however, the rate of sugar loading of LZM increased sharply from 3.1 to 6.8 between the 5th and 10th days of incubation, compared with 2.9 to 4.2 in the absence of oxygen. The presence or absence of oxygen did not show an apparent effect on the initial rate of sugar loading of LZM in the LZM-F system, although the overall sugar loading of

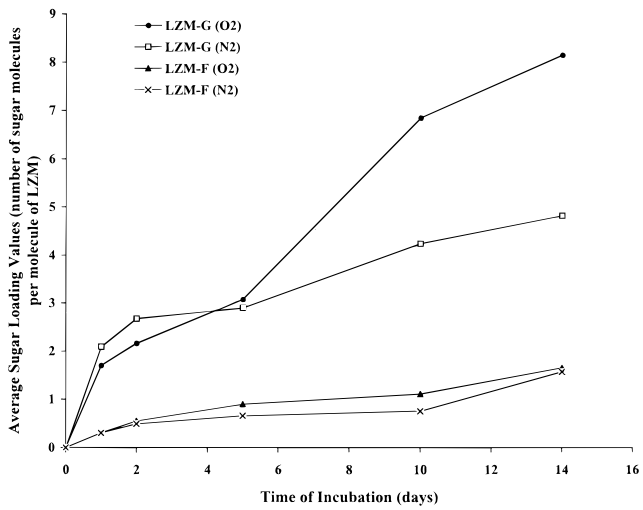


Figure 2. Graph of the average sugar loading values (SLV) of lysozyme with time of incubation as determined from the ESI-MS spectra.

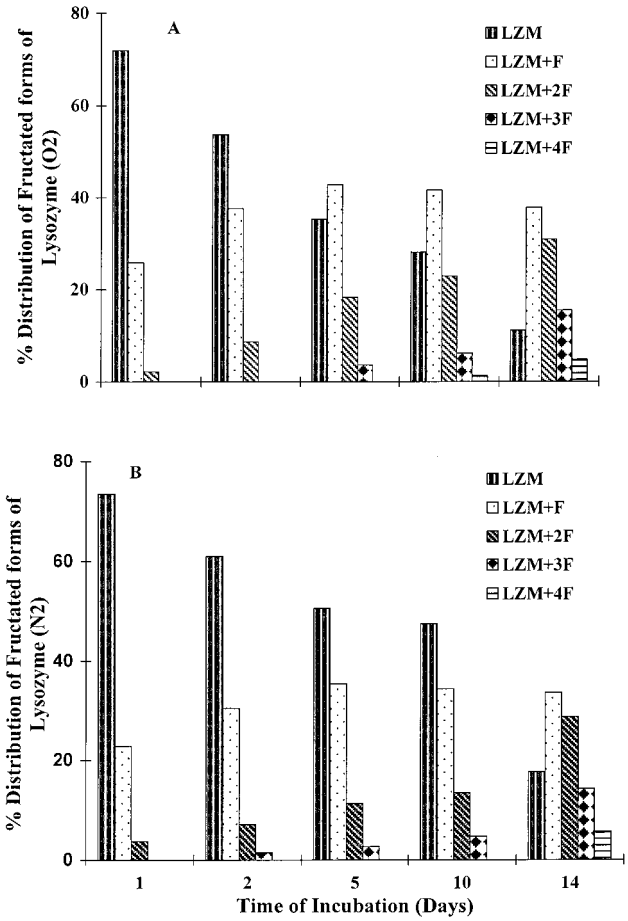


Figure 3. Percentage distribution of the glycoforms of lysozyme in the lysozyme−D-fructose (LZM-F) system incubated (A) in the presence of oxygen and (B) in the absence of oxygen over a 14 day incubation period.

LZM in the LZM-F system was slightly higher in the presence of oxygen.

Distribution of the Major Groups of Glycoforms of LZM. The percentage distribution of the different glycoforms of monomeric LZM in the incubation mixtures at different stages of incubation is presented in Figures 3 and 4. Panels A and B represent LZM-F samples incubated in the presence and in the absence

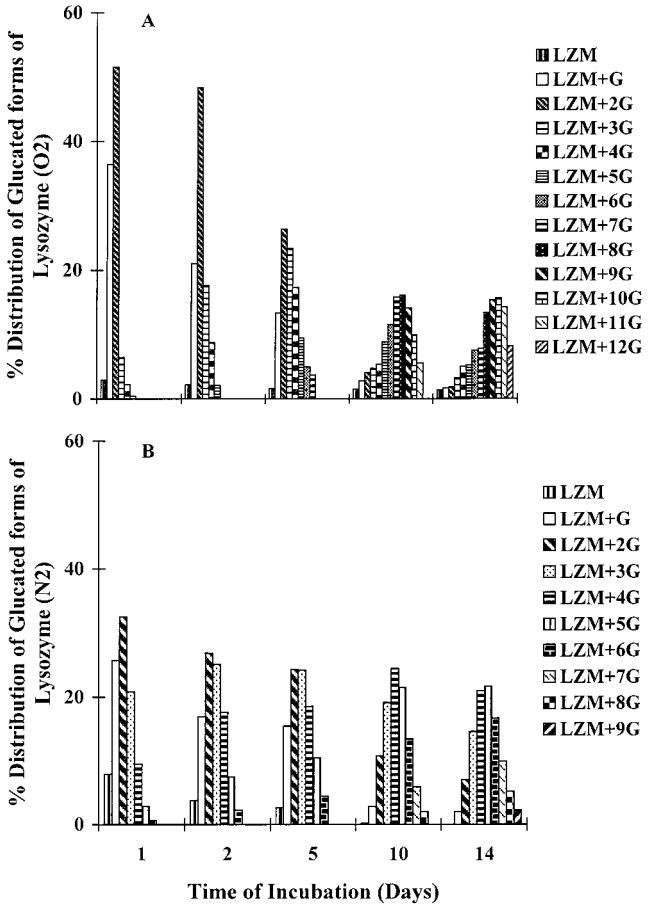


Figure 4. Percentage distribution of the glycoforms of lysozyme in the lysozyme−D-glucose (LZM-G) system incubated (A) in the presence of oxygen and (B) in the absence of oxygen over a 14 day incubation period.

of oxygen, respectively. A comparison of panels A and B of Figure 3 reveals some important differences between the glycation of LZM in the presence and in the absence of oxygen. By the second day of incubation in the absence of oxygen, glycated LZM containing up to three fructose moieties could be observed, whereas in the presence of oxygen the highest glycated form of LZM observed was difructated LZM. It can also be observed that by the fifth day of incubation, the relative proportion of monofructated LZM in the LZM-F system incubated in the presence of oxygen had exceeded that of intact LZM in the incubation mixture. This did not occur in the LZM-F system incubated in the absence of oxygen until the 14th day of incubation. In general, the proportion of glycoforms of LZM with increasing number of attached fructose moieties increased with incubation, and by the 14th day of incubation, fructated LZM containing up to four fructose moieties could be identified in the LZM-F systems.

A comparison of panels A and B of Figure 4 also shows a distinct difference in the distribution of the glycoforms of LZM during the incubation of LZM with glucose in the presence and in the absence of oxygen. By the first day of incubation, glycated LZM with up to six glucose moieties was observed in the absence of oxygen, compared with five glucose moieties for the sample incubated in the presence of oxygen. In addition, the proportion of multiglycated LZM (tri-, tetra-, penta-, and hexa-) was higher in the LZM-G system incubated in the absence of oxygen. By the 5th day of incubation, however, higher glycoforms of LZM (up to seven glucose

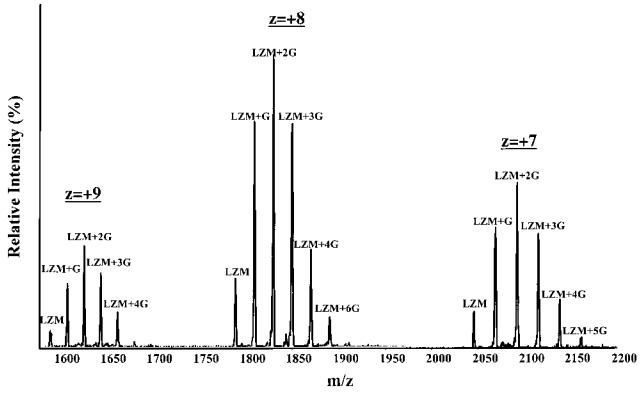


Figure 5. Typical electrospray mass spectrum of glycated lysozyme, showing a heterogeneous distribution of glycoforms at different ionization states (+7, +8, and +9). The figure represents the ESI-MS spectrum of LZM-G after 1 day of incubation in the absence of oxygen.

moieties) could be observed in the LZM-G system incubated in the presence of oxygen than in its absence. By the 10th and 14th days of incubation, LZM containing up to 11 and 12 glucose moieties, respectively, could be observed in the LZM-G system incubated in the presence of oxygen, compared with 8 and 9 glucose moieties, respectively, in the LZM-G system incubated in the absence of oxygen. The results also show that the distribution profile of the glycoforms of LZM in the LZM-G system incubated in the absence of oxygen showed a normal distribution with time of incubation, whereas that in the presence of oxygen showed a skewed distribution toward higher glycoforms with time of incubation. Comparison of the LZM-F and LZM-G systems revealed that the LZM-G system showed a higher level of glycation and a wider distribution of glycoforms at all stages and conditions of incubation.

Dehydration of Amadori and Heyns Products of LZM. Figure 5 is a typical ESI-MS spectrum of glycated LZM. The figure represents the ESI-MS spectrum of glycated LZM after the first day of incubation with D-glucose in the absence of oxygen. The results show a distribution of different glycoforms of LZM at different multiply charged states (+7, +8, and +9). Each peak in a cluster at a particular charged state represents a glycoform of LZM (Amadori product) containing a specific number of sugar moieties. The molecular mass difference between adjacent molecular ion peaks within a cluster was determined to be 162 atomic mass units (amu), which is equivalent to the mass of a covalently bound hexose moiety.

Panels A, B, C, and D of Figure 6 represent the molecular ions of a cluster of glycoforms associated with the +9 charged state of LZM and glycated LZM incubated in the absence of oxygen after 1, 2, and 10 days, respectively. The results show the appearance of new molecular ion peaks on the left shoulder of the molecular ions of the Amadori products with time of incubation. The molecular ions represented by the adjacent shoulder peaks are separated from each other by 18 amu, which indicates successive loss of water molecules from the Amadori products. The results also show a progressive decrease in the proportion of intact LZM in the glycation mixture and an increase in the heterogeneous distribution of glycoforms of LZM (Morgan et al., 1998). In addition, a shift in the distribution of glycoforms toward higher glycoconjugates (increasing number of attached sugar moieties) with time of incubation can be observed.

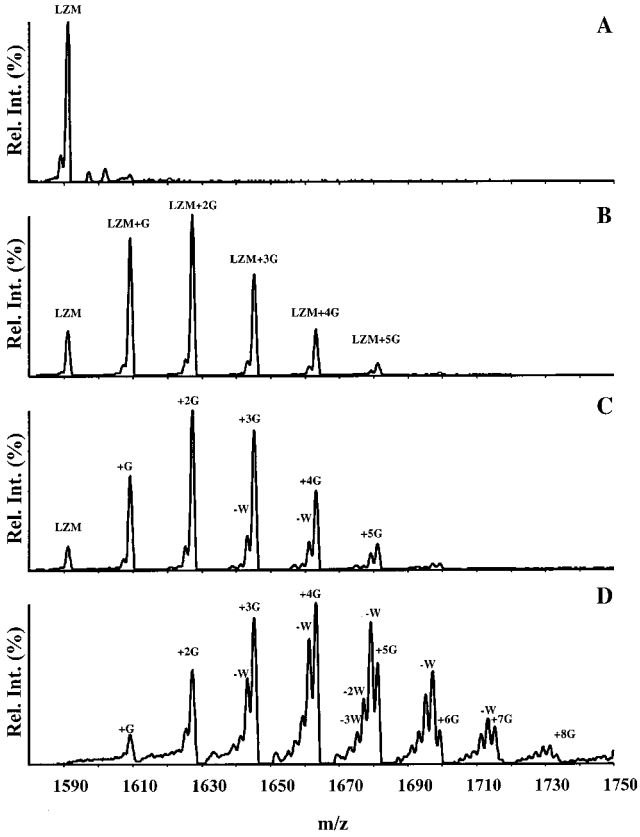


Figure 6. Mass spectrum of (A) unmodified lysozyme (LZM) and the lysozyme−D-glucose mixtures (LZM-G) incubated in the absence of oxygen after (B) 1 day, (C) 2 days, and (D) 14 days of incubation. LZM+nG represents glycated lysozyme containing *n* sugar moieties, and (-nW) represents Amadori products of LZM that has lost *n* water molecules.

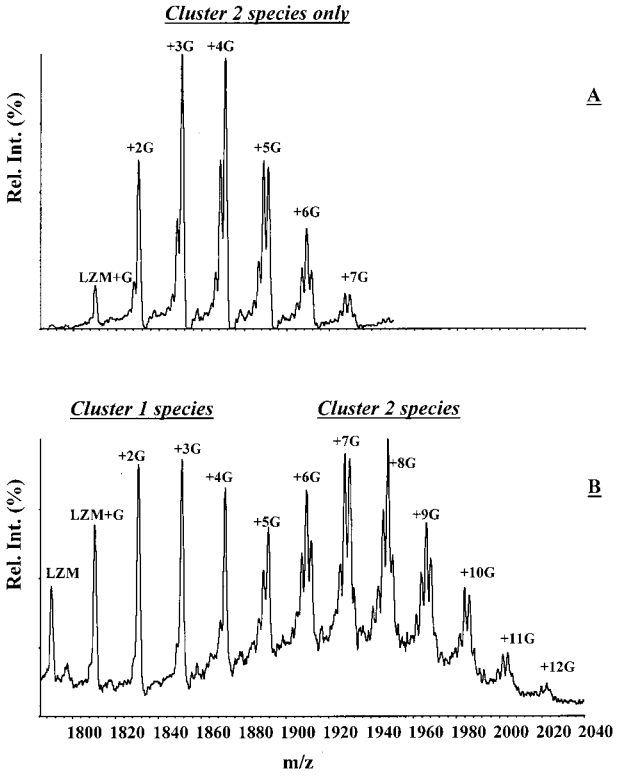


Figure 7. Electrospray mass spectrum of LZM-G after 10 days of incubation (A) in the absence of oxygen (showing a single cluster of normally distributed glycoforms) and (B) after 10 days of incubation in the presence of oxygen (showing two distinct normally distributed clusters of glycoforms).

It can also be seen that the intensity and the number of shoulder peaks associated with the loss of water molecules from molecular ions of the Amadori products increased with time of incubation and increasing sugar load.

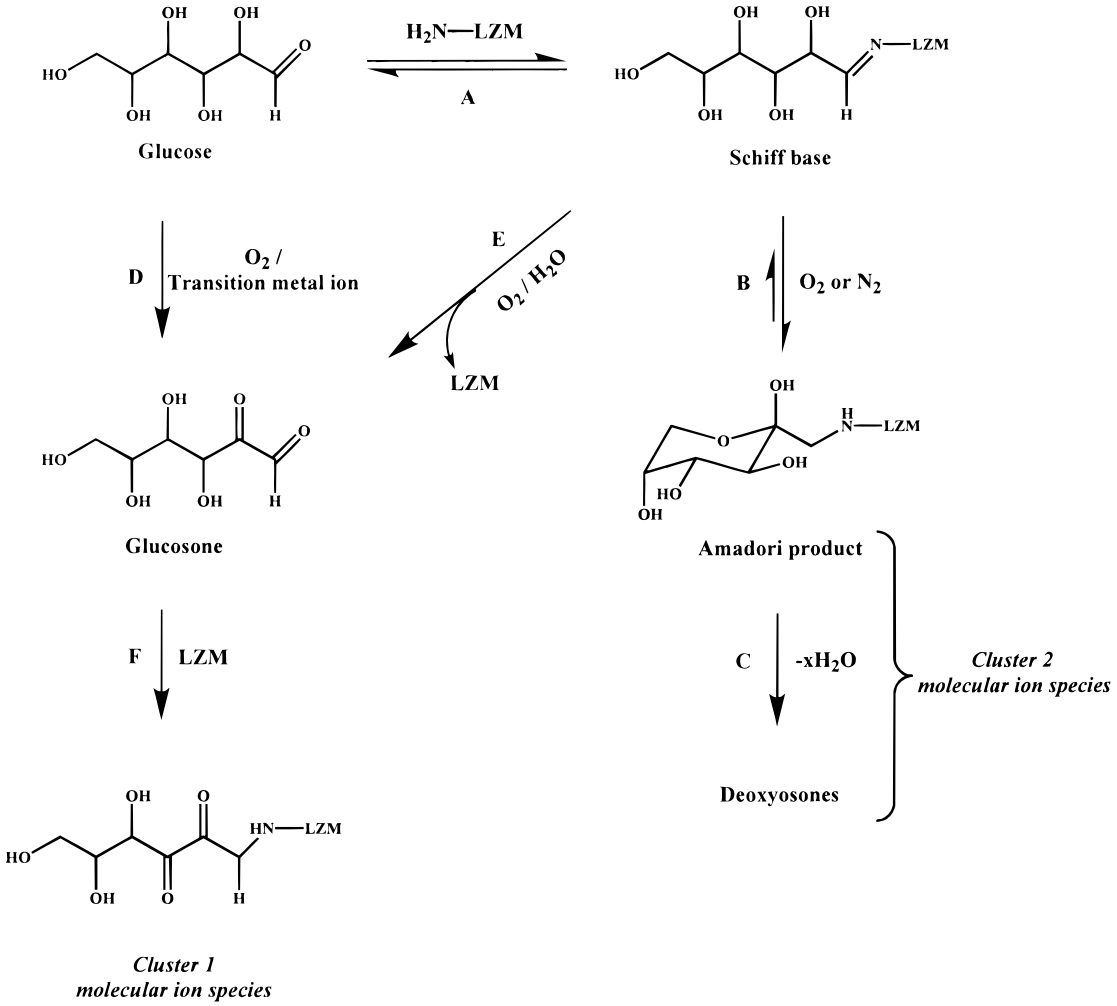
Effect of Oxygen on the Distribution of Glycoforms. Panels A and B of Figure 7 show the cluster of molecular ions associated with the +8 charged state of the LZM-G system after 14 days of incubation in the absence and in the presence of oxygen, respectively. The ESI-MS spectrum show a single cluster of normally distributed glycoforms of LZM for the LZM−glucose mixture incubated in the absence of oxygen, whereas in the presence of oxygen the mass spectrum showed two distinct overlapping clusters of normally distributed glycoforms of LZM (molecular ion *clusters* 1 and 2 in Figure 7B). The distribution profile of the glycoforms of LZM (see Figures 3 and 4) also shows that the presence of oxygen in the incubation systems influenced the distribution of glycoforms, at both the early and advanced stages of incubation. In the presence of oxygen, a narrower range of distribution of glycoforms of LZM was observed during the early stages of incubation (first and second days), whereas a wider range of distribution of glycoforms was observed during the latter stages of incubation.

DISCUSSION

Fluorescamine Assay versus Mass Spectrometric Evaluation of Glycation. The results of the fluorescamine assay (Figure 1) confirm the reports of previous studies (Yeboah et al., 1999; Naranjo et al., 1998; Baxter, 1995) that under low moisture conditions, D-glucose is more reactive than D-fructose, with respect to their ability to react with the ϵ-amino groups of proteins. The results are also in agreement with the conclusions of our previous study (Yeboah et al., 1999), which reported that the presence of oxygen in the reaction system influences the initial rate of formation of Amadori products by inducing a competitive glycoxidative side reaction during the initial stages of incubation. The rate of glycation as determined by the average sugar loading values of LZM (SLV) (Figure 2) shows a different profile than the rate of glycation determined by the fluorescamine assay. The main difference between the fluorescamine assay and the SLV is that the former measures the average number of lysine residues of LZM that have been glycated, whereas the latter measures the average number of sugar moieties per molecule of LZM. In addition, the fluorescamine assay cannot distinguish between molecules at different stages of the glycation reaction, such as the Amadori stage and the subsequent degradation reactions.

A comparison between Figures 1 and 2 shows that the rates of glycation of LZM as measured by the fluorescamine assay tapered off after the early stages of incubation (first and second days), whereas the rate of glycation as determined by the SLV increased during the latter stages of incubation. This can be explained by the fact that the fluorescamine assay measures, essentially, the glycation of the ϵ-amino groups of LZM and, possibly, the N-terminal α-amino group. Because the rate of glycation of the ϵ-amino groups of LZM is dependent on their concentration and reactivity, the initial rate of glycation (as determined by the fluorescamine assay) will be high, when the concentration of

Scheme 1. Proposed Pathways of Early Glycation of Amadori and Schiff Adducts of Lysozyme under Aerobic and Anaerobic Conditions and the Origin of Cluster Ions 1 and 2 Observed under ESI-MS Conditions



the ϵ-amino groups is highest and the reducing sugars are glycating the most reactive amino groups. During the advanced stages of incubation, when the concentration of ϵ-amino groups is low and sugars are reacting with the least reactive ϵ-amino groups, the rate of glycation of the ϵ-amino groups of LZM will be low. The rate of sugar loading of LZM, on the other hand, is dependent on the concentration and reactivities of all the potential glycation sites of LZM, including the N-terminal α-amino group, the ϵ-amino group of lysine residues, and the guanidino group of arginine residues (Tagami et al., 2000). The initial rate of sugar loading of LZM will, however, depend on the concentration of the ϵ-amino groups, as they are the most susceptible to glycation. The results of the average number of glycated ϵ-amino groups of LZM by 14th day of incubation in the absence of oxygen in both the LZM-G and LZM-F systems (3.8 and 1.7, respectively) are consistent with the corresponding results of the SLV of LZM (4.8 and 1.6, respectively). During the latter stages of incubation, especially in the presence of oxygen, the dicarbonyl compounds (such as glucosone, which are more reactive than the original reducing sugars) generated in the reaction system through glycoxidation also participate in the reaction and increase the rate of glycation. The more reactive glycoxidation products (dicarbonyls such as glucosone) also have a higher affinity for the guanidino groups of arginine residues and hence increase the involvement of arginine residues in the glycation reaction (see Scheme 1). The differences observed in the rates of glycation of LZM by D-glucose or by D-fructose, especially in the presence of oxygen during the latter stages of incubation, may be explained by the higher susceptibility of glucose to glycoxidation under dry heating conditions.

The results also show that the highest SLV of LZM in the LZM-F system was 1.6, which is far less than the number of ϵ-amino groups of LZM (six ϵ-amino groups), suggesting that fructose reacted mainly with the most reactive ϵ-amino groups of lysine residues of LZM. The SLV of LZM in the LZM-G system, on the other hand, exceeded the number of ϵ-amino groups by the 10th day of incubation (6.8 mol/mol) in the presence of oxygen. The high SLV in the LZM-G system indicates that glucose reacted with all of the primary amino groups of LZM, as well as some of its guanidino groups. The high SLV may also be explained by diglycation at some of the ϵ-amino groups of LZM (Blakytny et al., 1997; Prabhakaram et al., 1996). These results also indicate that the involvement of other amino acid residues in the glycation reaction may be influenced by, among other things, the nature of the interacting reducing sugar and the presence of oxygen.

Distribution of the Glycoforms of Lysozyme. A comparison between the distribution profiles of the glycoforms of LZM in the LZM-G system (Figure 4A,B) suggests that the natures of the glycation reaction in the presence and absence of oxygen are different. During

the first day of incubation in the presence of oxygen, mono- and diglycated LZM were preferentially formed over the higher glycoforms of LZM (i.e., tri-, tetra-, pentaglycated LZM, etc.) when compared with that in the absence of oxygen. In the presence of oxygen, mono- and diglycated LZM species constituted >88% of LZM, compared with 58% in the absence of oxygen. This suggests that the presence of oxygen in the reaction system induced some level of selectivity in the glycation of the amino groups of LZM. The oxygen-induced selectivity may arise from the fact that the reducing sugars can undergo other competing side reactions, such as glycoxidation (Yaylayan et al., 1994; Wells-Knecht et al., 1995; Ortwerth et al., 1998), in the presence of oxygen and trace amounts of transition metal ions. These competing side reactions involving the reducing sugars will diminish their effective concentration in the reaction system that is available to react with the amino groups of LZM. Considering that the reducing sugar is limiting in the reaction system, only the most reactive amino groups of lysozyme will undergo glycation during the early stages of incubation, thus introducing some level of selectivity in the glycation reaction. In the absence of oxygen, on the other hand, there are no oxidative side reactions involving the reducing sugars, so that all of the sugar is available to glycate the amino groups of LZM, resulting in the formation of a wider range of glycoforms of LZM with a normal distribution. The selectivity observed in the presence of oxygen was more pronounced in LZM-G system when compared with the LZM-F system. This is due to the higher tendency of glucose (an aldose) to undergo oxidation than fructose (a ketose). The effect of fructose oxidation in the presence of oxygen will therefore have a relatively small effect on the effective concentration of fructose during the early stages of the reaction. After the 5th day of incubation, however, the level of glycation of lysozyme in the presence of oxygen exceeded the level of glycation in its absence (Figures 3 and 4). The accelerated rate of glycation during the intermediate and advanced stages of the reaction may be explained by the increased accumulation of more reactive oxidation products of the reducing sugars (such as glucosone) formed during the early stage of the reaction (Yeboah et al., 1999).

Effect of Oxygen on the Profile of Glycoconjugates of Lysozyme. The differences observed between the glycoconjugate profiles of LZM in the presence and absence of oxygen can be ascribed to autoxidation of the reducing sugars and oxidative cleavage of the Schiff base intermediates during the early stage of the reaction in the presence of oxygen (Scheme 1). At the onset of incubation and in the absence of oxygen, the glycation reaction proceeds through path A to form a Schiff base, which rearranges (path B) to the more stable ketosamine (Amadori product). Further dehydration (path C) of the Amadori product will yield the deoxyosone derivatives (only cluster 2 species). In the presence of oxygen and trace transition metal ions, the reducing sugars can undergo autoxidation to form α-dicarbonyls such as glucosone (path D). The intermediate Schiff base can also undergo oxidative cleavage (path E) to form dicarbonyl compounds (Hofmann et al., 1999). The more reactive dicarbonyls can react with both the primary amino and guanidino groups of glycated or unglycated LZM (path F) and increase the rate of glycation, especially during the latter stages of incubation. The interaction between LZM and the glycoxidation products (path F) will also produce a new class of glycoconjugates of LZM (*cluster* 1 glycated species, see Figure 7B). The formation of this new class of glycoconjugates was more apparent in the LZM-G system incubated in the presence of oxygen after the fifth day of incubation, where two distinct distributions of clusters of glycated species of LZM were observed (Figure 7B). The molecular mass difference between the molecular ions of the glycoconjugates of LZM in *cluster* 1 (160 amu) is consistent with the formation of glucosone from the initial oxidation of glucose (Hofmann et al., 1999).

As mentioned previously, the competing oxidative reactions in the reaction system during the early stage of incubation in the presence of oxygen will reduce the initial rate of glycation, relative to that in the absence of oxygen, especially in the LZM-G system (clusters 1 and 2).

The higher level of dehydration of the sugar moieties of di-, tri-, and tetraglycated LZM species in the absence of oxygen (Figure 7A), compared with the species of similar level of glycation in the presence of oxygen (*cluster* 1, Figure 7B) may be due to the following reasons: (a) the di-, tri-, and tetraglycated species of LZM in the presence of oxygen are newly formed, through the interaction between LZM and glucosone, so that they have not had time to undergo dehydration or (b) the dehydration products of the 2,3-diulose derivative of Amadori rearrangement product formed through the interaction between lysozyme and glucosone are very reactive (tricarbonyl), so that they did not form at all or accumulate in the reaction system to an appreciable extent.

CONCLUSION

The results of this study clearly demonstrate the higher reactivity of D-glucose, compared with D-fructose, in undergoing condensation reactions with amines under dry heating conditions. The lower reactivity of fructose limited its interaction to the most reactive ϵ-amino groups of LZM, resulting in a low degree of glycation and a narrow distribution of glycoforms. The presence of oxygen in the reaction system at the initial stage of the glycation reaction resulted in a parallel, competitive glycoxidation of the reducing sugars and/or the intermediate Schiff bases. This reduced the initial rate of formation of Amadori/Heyns products in the samples incubated in the presence of oxygen. The reduction of the effective initial concentration of the reducing sugars also resulted in some level of the selectivity in the glycation of the most reactive ϵ-amino groups of LZM during the initial stages of incubation.

The results of this study also suggest that under dry heating conditions of glycation, glucosone is the most likely glycoxidation product of glucose during the early stage of glucose oxidation in the presence of oxygen. The involvement of an arginine residue in the glycation reaction during the latter stages of incubation in the presence oxygen also suggests that arginine residues have a higher affinity for α-dicarbonyls than for aldehydes. The study also confirms that the dehydration of Amadori and Heyns products to form deoxydicarbonyl compounds is the onset of their degradation and their major degradation pathway.

The very extensive nature of the glycation reaction in the LZM-G system, even after only 1 day of incubation, under the experimental conditions show the ease

with which protein glycation can occur. It is therefore important to carefully control and monitor food-processing and storage conditions of foods, for which reducing sugars and proteins constitute a major part of the ingredients and for which extensive Maillard reaction is not desirable. It is also important to regulate the oxygen content in the atmosphere of packaged low-moisture, high-protein, and high-sugar foods, such as infant formulas, to minimize protein−sugar interactions and improve product stability.

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