Direct Observation of Protein Glycosylation by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Nonenzymatic glycosylation products of several peptides and lysozyme were observed directly by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) operating in a linear mode, and the number of glucose units added was determined from the observed mass. Oxidation products of the peptides and the glycosylated peptides were also observed. Lysine residues were readily glycosylated; however, a nonapeptide without lysine residue also showed addition of up to four glucose units, suggesting that other amino acid residues can also be glycosylated. The MALDI-TOF-MS technique was useful for comparing reaction products from the glycosylation of different peptides as well as for demonstrating inhibition of glycosylation by *N*-α-acetyl-L-lysine.

Keywords: *MALDI-TOF-MS; nonenzymatic glycosylation; Maillard reaction; glucagon; lysozyme*

INTRODUCTION

Nonenzymatic glycosylation is the initial step in a series of complex reactions involving reducing sugars and amines. In foods, such reactions are collectively known as the Maillard reaction. The Maillard reaction is important in the development of food color and flavor. Late-stage Maillard reaction could lead to cross-linking of proteins, which might result in decreased nutritive value and alteration of the physicochemical properties of the proteins (Tanaka et al., 1977; Kim et al., 1984; Kato et al., 1995). By minimizing conditions that facilitate glycosylation, it is possible to optimize product quality both initially and after long-term storage. Nonenzymatic glycosylation also leads to advanced-glycation end-products (AGEs) under physiological conditions. AGEs are implicated in aging, Alzheimer's disease, and complications in diabetic patients such as cataracts and hypertension (Porte and Schwartz, 1996; Vasan et al., 1996; Bucala and Cerami, 1992; Brownlee et al., 1986).

The protein glycosylation reaction is difficult to monitor, and there seems to be no generally applicable technique to analyze for glycosylated proteins (Ledl and Schleicher, 1990). Methods for the quantitative analysis of glycosylated proteins are indirect and involve acid hydrolysis, oxidation, reduction, or chemical labeling followed by chromatographic or spectrophotometric analyses. Glycosylated hemoglobins were chromatographically separated as early as 1958 (Allen et al., 1958); however, chromatographic analysis does not provide mass information about the glycosylated proteins. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) can be used to detect dimers and trimers of proteins formed through glycosylation (Okitani et al., 1984). However, the mass accuracy of SDS−PAGE is limited to about 5%, and the slight increase in the molecular weight of the proteins upon glycosylation (approximately 1% when one molecule of glucose is added to a lysozyme molecule) cannot be detected by SDS−PAGE.

In 1988, Karas and Hillenkamp showed that proteins higher than 10 000 Da in mass can be desorbed and ionized by the transfer of laser energy through an organic matrix (Karas and Hillenkamp, 1988). The molecular weight of the ionized protein was determined from the flight time between the laser pulse and the arrival of the ion to the detector. Since the absorption of energy by the proteins is indirect, the peptide backbone remains intact and the molecular weight of the parent protein can be determined with a high accuracy. The technique became known as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Within several years, MALDI-TOF-MS has been established as a powerful technique for accurate molecular weight determination of biopolymers (Hillenkamp, 1994). Recently, detection of polymers up to 1.5 million Da was reported (Schriemer and Li, 1996). In 1995, a delayed extraction technique was added to MALDI-TOF-MS to counteract the initial velocity distribution of the sample ions (Vestal et al., 1995). Mass resolution, $M/\Delta M$ (full width at half-maximum), greater than 4000 and separation of isotopic peaks were reported for peptides (1000−3000 Da) (Vestal et al., 1994; Whittal and Li, 1995).

Application of MALDI-TOF-MS to food science has just begun (Sporns and Abell, 1996). Recently, MALDI-MS was used for analysis of glutenin (Hickman et al., 1995), fruit juice carotenol fatty acid esters and carotenoids (Wingerath et al., 1996), potato glycoalkaloids (Abell and Sporns, 1996), and peptides released by trypsin from casein micelles (Diaz et al., 1996). In this paper, we show that the MALDI-TOF-MS technique can be used to directly observe glycosylated peptides with different numbers of glucose molecules and oxygen atoms bound and, thereby, to assess the susceptibility of the peptides toward glycosylation and oxidation. We also show that it can be utilized to demonstrate the

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efficacy of an approach to inhibit nonenzymatic glycosylation and subsequent cross-linking.

MATERIALS AND METHODS

Reagents. Lys-Trp-Lys (tripeptide, acetate salt), Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu (nonapeptide), glucagon, lysozyme (chicken egg white), trypsin, and *N*-α-acetyl-L-lysine were from Sigma (St. Louis, MO). D-Glucose was from Aldrich (Milwaukee, WI).

Glycosylation Reaction. A 0.2 mL aliquot of an aqueous solution containing 14.9 mM tripeptide and 0.67 mM nonapeptide was evaporated to dryness in an open vial (to promote oxidation of certain amino acid residues) by placing it in a convection oven preheated to 90 °C and holding it at that temperature for 2 h (sample A1). A 0.2 mL solution containing 28 mM D-glucose in addition to 14.9 mM tripeptide and 0.67 mM nonapeptide was similarly dried and heated for 30 min at the same temperature (sample A2).

A 0.2 mL aliquot of an aqueous solution of glucagon (0.71 mM) and glucose (10 mM) was heated for 1 h at 90 °C after drying (sample B). For preparation of sample C, glucagon (4 mg/mL in deionized water) was digested with trypsin (2% w/w) for 24 h at 40 °C. The digest was filtered through a 0.45 μm membrane filter (Alltech, Deerfield, IL). An aliquot of the digest (sample C1) was analyzed by MALDI-MS without heating. A 0.2 mL aliquot containing 0.55 mM glucagon digest and 12 mM glucose was heated for 1 h at 90 °C after drying as above (sample C2). For sample D1, a 0.2 mL aqueous solution containing 0.21 mM lysozyme and 56 mM glucose was heated similarly for 1 h at 90 °C after drying. For sample D2, 53 mM *N*-α-acetyl-L-lysine was added to the lysozyme and glucose in sample D1 and then evaporated and heated next to sample D1 in the oven. All dried samples were redissolved in 0.2 mL of deionized water and diluted 10-fold for mass spectrometric analysis. The tripeptide, glucagon, and lysozyme were also analyzed without heat treatment.

MALDI-TOF-MS. Time-of-flight mass spectra were obtained using a Voyager Biospectrometry workstation with linear mass analyzer (PerSeptive Biosystems, Framingham, MA), which employs a 337 nm nitrogen laser and a 1.2 m linear flight tube. Positive ion spectra were collected at a 30 kV accelerating voltage, and typically about 120 scans were averaged. The system was calibrated with angiotensin I (1296.5 Da) and ACTH clip (18−39) (2465.7 Da) for peptides and with a mixture of cytochrome *c* (12358.2 Da), equine cardiac myoglobin (16950.5 Da), and bovine serum albumin (66429.2 Da) for lysozyme.

A half microliter of the sample solution was mixed with another half microliter of a matrix solution (10 mg of the matrix compound/mL of a 1:1 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid) on the sample plate and allowed to air-dry. The matrix compounds used were α-cyano-4-hydroxycinnamic acid for peptides and sinapinic acid for lysozyme.

RESULTS AND DISCUSSION

Tripeptide (TP) and Nonapeptide (NP). The top panel of Figure 1 shows the tripeptide (460.6 Da) portion of the mass spectrum obtained from sample A1, tripeptide and nonapeptide dried at 90 °C without glucose as a control. Two major species observed at *m*/*z* of 460.8 and 483.0 correspond to $(TP + H)^+$ and $(TP + Na)^+$, respectively. The mass accuracy was within 0.2%. $(TP + Na)^+$ was observed due to the sodium presumably present in the acetate salt of the tripeptide. A smaller peak at *m*/*z* 504.9 probably corresponds to $(TP + 2Na)^+$. Several species containing oxygen atom were observed [*m*/*z* 476.8, $(TP + O + H)^+$; 498.8, $(TP + O + Na)^+$; 520.8, $(TP + O + 2Na)^+$; 492.9, $(TP + 2O + H)^+$; 515.1, $(TP + 2O + Na)^+$; and 537.5, $(TP + 2O + 2Na)^+$]. Analysis of the tripeptide without heat treatment showed only $(TP + H)^+$ and $(TP + Na)^+$.

The bottom panel of Figure 1 shows the nonapeptide (848.8 Da) portion of the mass spectrum obtained from sample A1. Again, two major species observed at 849.3 and 871.2 Da correspond to $(NP + H)^+$ and $(NP + Na)^+$, respectively. The smaller peaks at 893.0 and 915.4 Da appear to correspond to $(NP + 2Na)^+$ and $(NP + 3Na)^+$, respectively. Oxidized species were also observed [886.8 Da, $(NP + O + Na)^+$; and 908.7 Da, $(NP + O + 2Na)^+$]. The oxidized species were not observed from untreated peptides. Oxidation is believed to take place at the tryptophan residue in both peptides during evaporation of the solution.

Figure 2 shows reaction products from these peptides when heated in the presence of glucose (sample A2). Little of the tripeptide and nonapeptide remained; however, several glycosylated species were observed, which are believed to be $(TP + G + H)^+$ (623.0 Da), $(TP + 2G + H)^+$ (785.4 Da), $(NP + G + H)^+$ (1012.1 Da), $(NP + 2G + H)^+$ (1174.9 Da), $(NP + 3G + H)^+$ (1337.4 Da), and $(NP + 4G + H)^+$ (1499.7 Da). The broad background indicates the presence of a variety of other reaction products.

The addition of two glucose molecules to the tripeptide, Lys-Trp-Lys, presumably takes place at the two ε-amino groups of the lysine residues. It appears that the tripeptide with three glucose units (expected at 947.6 Da), where the third glucose would be bound to the α-amino group on the N-terminal lysine, is at a much lower concentration, if at all, compared with the tripeptide with one or two glucose units and is buried in the background (Figure 2). This observation might imply that the two ε-amino groups of the lysine residues are more reactive than the α-amino group.

Observation of the nonapeptide with one to four glucose units was unexpected. The only amino group in the nonapeptide is the α-amino group on the N-terminal tryptophan, and it cannot account for addition of four glucose molecules. In addition to lysine, arginine (Mohammad et al., 1949; Clark and Tannenbaum, 1974) and tryptophan (Cho et al., 1984) in proteins are known to be significantly impaired by the nonenzymatic browning reaction with glucose. Thus, the only reactive residue on the nonapeptide appears to be tryptophan, which is the N-terminal amino acid. It would be interesting to investigate whether the proximity of the amino group and the indole ring of the N-terminal tryptophan could explain the addition of four glucose units possibly through condensation (Clark and Tannenbaum, 1974).

The mass spectrometric results enabled us to qualitatively observe the difference in susceptibility to glycosylation between the tripeptide and the nonapeptide. Initially, a 22-fold molar excess of tripeptide over nonapeptide was present in the reaction mixture. The signal intensities for the tripeptide and the nonapeptide in Figure 1 are consistent with the initial concentrations and indicate that peptides in a mixture can be observed independently by MALDI-TOF-MS. (The top and bottom mass spectra show different mass ranges from the same experiment at different *y*-scales. The signal intensity is not exactly proportional to the concentration in MALDI-MS due to the difference in desorption/ionization efficiency.) In Figure 2, the signal intensities of the nonapeptide adducts (NP + G, NP + 2G, NP + 3G, NP + 4G) are higher than those of the tripeptide adducts (TP + G, TP + 2G). It appears that the reaction of the more susceptible tripeptide has progressed beyond the initial glycosylation reaction to yield numerous

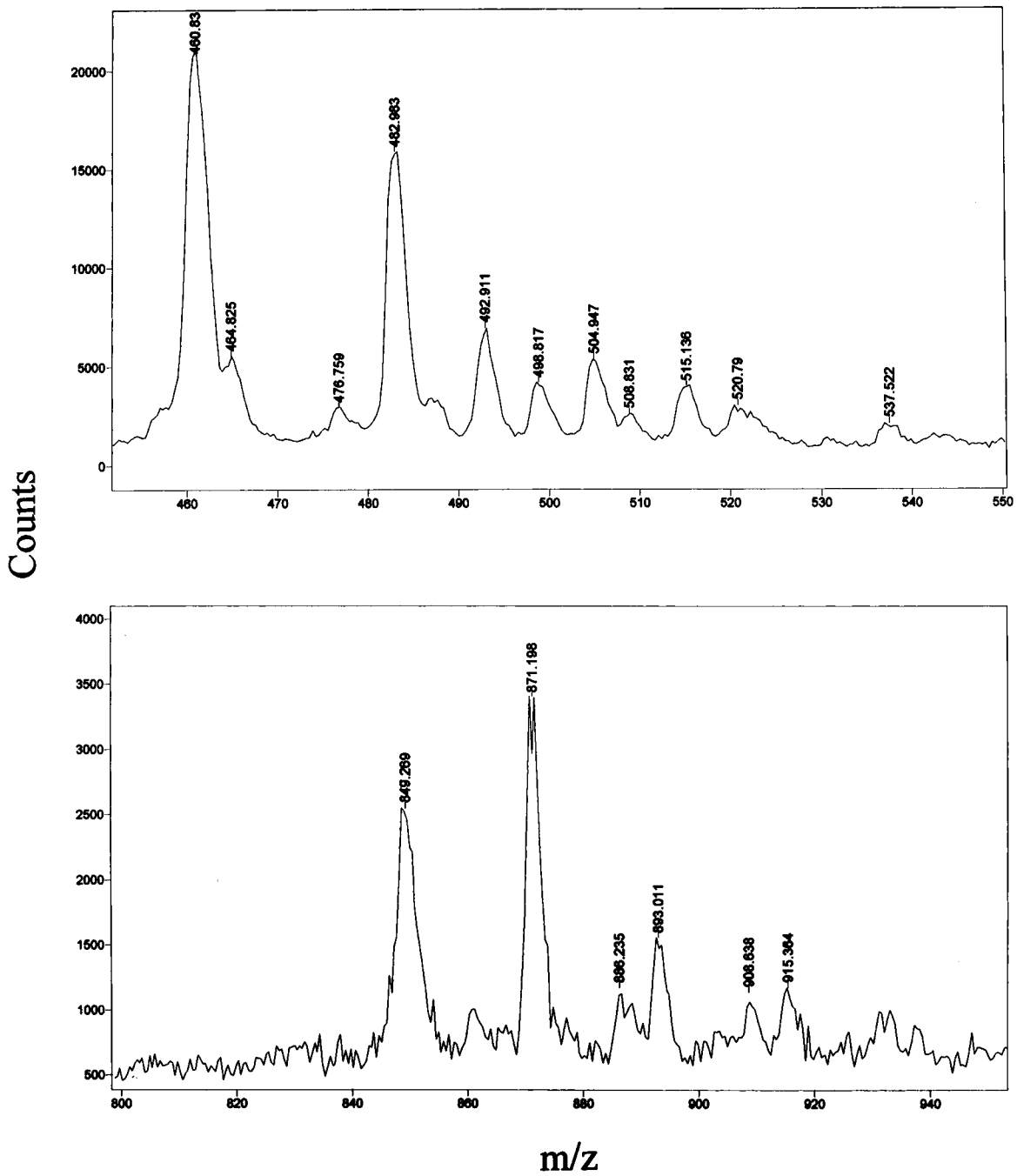

Figure 1. Tripeptide and nonapeptide portion of a TOF mass spectrum obtained from a mixture of these peptides dried at 90 °C. Peaks at 460.8, 483.0, and 504.9Da correspond to $(TP + H)^+$, $(TP + Na)^+$, and $(TP + 2Na)^+$, respectively; peaks at 476.8 and 492.9 Da correspond to $(TP + O + H)^+$ and $(TP + 2O + H)^+$, respectively (top). Peaks at 849.3 and 871.2 Da correspond to $(NP + H)^+$ and $(NP + Na)^+$, respectively (bottom).

advanced Maillard products, whereas the less susceptible nonapeptide is still at the initial glycosylation stage.

It was also noted that the glucose adducts in Figure 2 are preceded by smaller peaks 18 Da less in mass, presumably representing dehydration products. For example, a peak at 1481.7 Da seems to be a dehydration product of $(NP + 4G + H)^+$ (1499.7 Da). Whereas the nonapeptide adducts show only one dehydration product, the tripeptide adducts seem to show a series of dehydration products. For example, $(TP + 2G + H)^+$ (785.4 Da) is associated with 767.6 $(TP + 2G - H_2O + H)^+$, 750.7 $(TP + 2G - 2H_2O + H)^+$, 731.5 $(TP + 2G - 3H_2O + H)^+$, and 714.3 Da $(TP + 2G - 4H_2O + H)^+$. Formation of several dehydration products from the tripeptide adducts compared with just one from the nonapeptide adducts supports the above observation that the tripeptide is more susceptible than the nonapeptide and has advanced to the later stage of the Maillard reaction. The broad background in Figure 2 might be due to various advanced reaction products from the tripeptide adducts.

Glucagon (GL). Glucagon consists of 29 amino acids, and the molecular weight is 3482.8 Da. In addition to the N-terminal amino group, it has one ϵ-amino group on the lysine residue at position 12. When untreated glucagon was analyzed, a major peak at 3480.3 Da (0.1% error) was observed for $(GL + H)^+$ (result not shown). Figure 3 shows the result of glycosylation in sample B. Only a small peak of the glucagon was observed at 3479.6 Da. Instead, three groups of glycosylated glucagon were observed. The

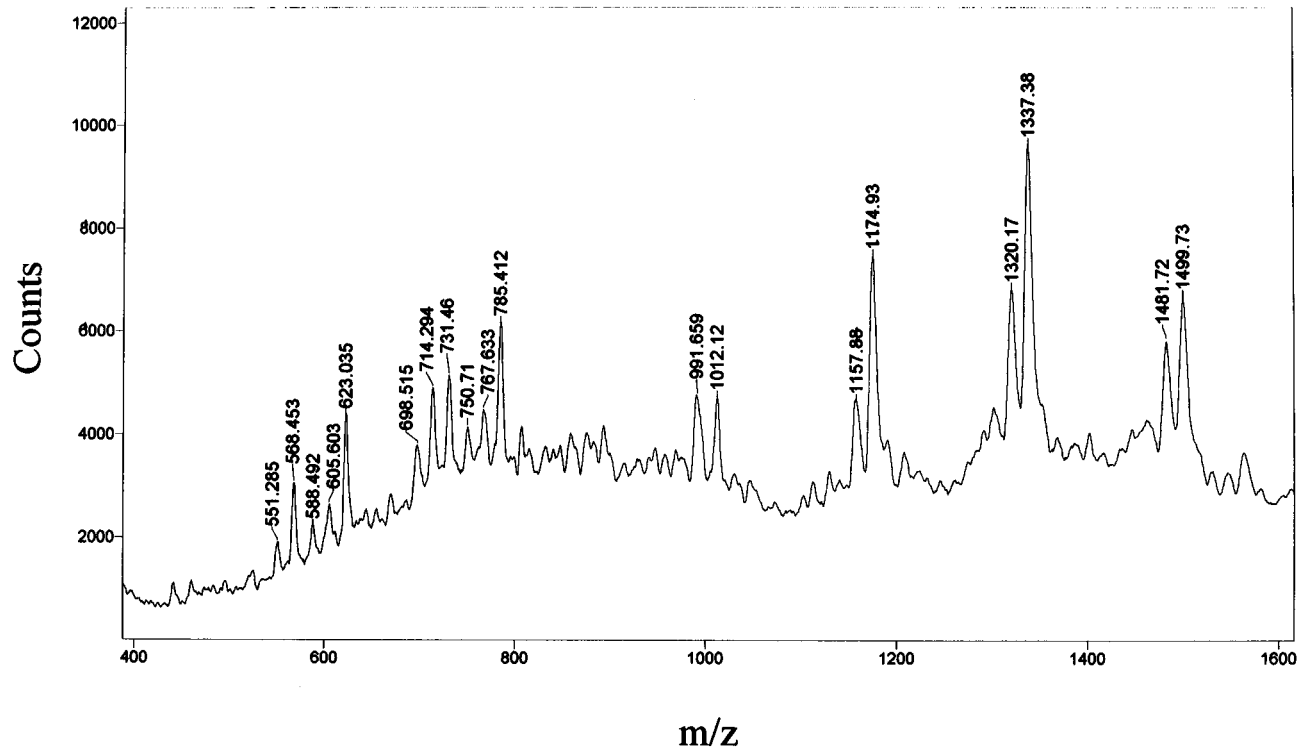

Figure 2. Mass spectrum obtained from a mixture of the tripeptide and the nonapeptide heated with glucose showing glucose adducts of the tripeptide (623.0, 785.4 Da) and of the nonapeptide (1012.1, 1174.9, 1337.4, 1499.7 Da). Dehydration products from these glucose adducts are also observed.

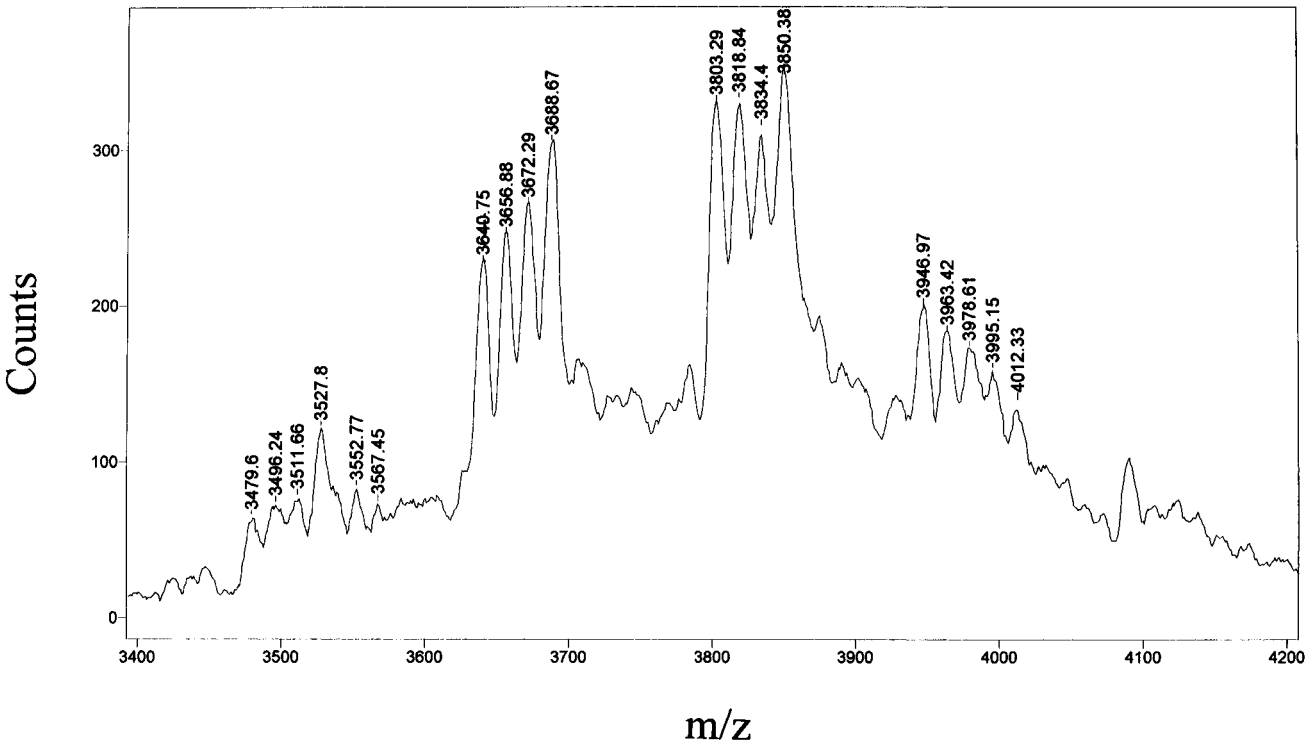

Figure 3. Mass spectrum showing glucose adducts of glucagon (3479.6, 3640.8, 3803.3, 3963.4 Da) and associated oxidation products.

peaks at 3640.8, 3803.3, and 3963.4 Da correspond to $(GL + G + H)^+$ (3645.9 ± 3.6 expected), $(GL + 2G + H)^+$ (3808.1 ± 3.8 expected), and $(GL + 3G + H)^+$ (3970.2 ± 4.0 expected), respectively.

Moreover, oxidation products from glucagon and its glucose adducts were clearly observed by MALDI-MS. For example, the mass difference between the four successive peaks starting with 3640.8 Da was 16.1, 15.4, and 16.4 (Figure 3). Again, the broad background suggests the formation of other modified adducts. Glucagon has two amino acid residues susceptible to oxidation, one tryptophan, and one methionine. It appears that three oxygen atoms are added between these two residues. The fact that the glucose adducts are oxidized as is glucagon suggests that the glycosylation sites and the oxidation sites are different on the glucagon.

Tryptic Digest of Glucagon. Trypsin hydrolyzes the peptide bond after lysine and arginine residues. The

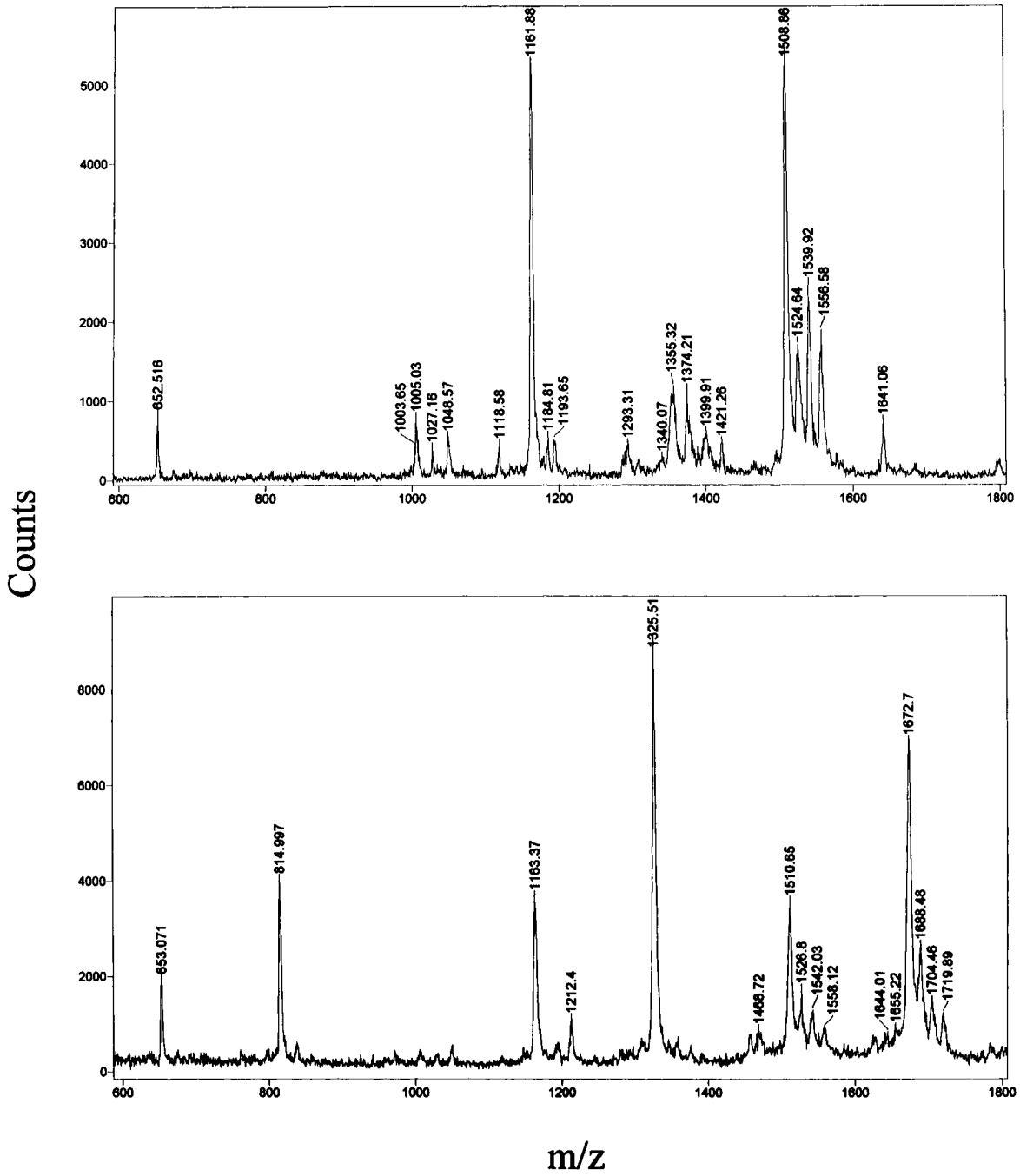

Figure 4. Mass spectrum showing peptides in the tryptic digest of glucagon (652.5, 1355.3, 1508.9 Da) (top) and glucose adducts of the tryptic peptides (815.0, 1672.7 Da) (bottom). Oxidation products are also observed.

peptides expected from tryptic digestion of glucagon are denoted below according to the position of the amino acid residues in the glucagon. Their predicted masses are shown in parentheses. Notice that the only lysine residue is in P_{1-12}.

P_{1-12}: His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys (1358.4 Da)

P_{13-17}: Tyr-Leu-Asp-Ser-Arg (652.7 Da)

Arg_{18}: (174.2 Da)

P_{19-29}: Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr (1352.5 Da)

When the tryptic digest of glucagon was subjected to MALDI-MS, $(P_{13-17} + H)^+$ was observed at 652.5 Da (Figure 4, top). Analysis of the peak at 1355.3 Da showed that it consists of a major peak at 1357.2 Da for $(P_{1-12} + H)^+$ and a smaller peak at 1352.6 Da for $(P_{19-29} + H)^+$. The mass observed at 1508.9 Da matched the mass expected for $(P_{18-29} + H)^+$, 1509.7 Da. It appears that trypsin does not readily recognize arginine when it is the N-terminal residue, and P_{18-29} instead of P_{19-29} is the primary peptide in the glucagon digest.

Several oxidation products of P_{18-29} such as $(P_{18-29} + O + H)^+$ (1524.6 Da), $(P_{18-29} + 2O + H)^+$ (1539.9 Da), and $(P_{18-29} + 3O + H)^+$ (1556.6 Da) were observed. P_{18-29} contains the tryptophan and methionine residues mentioned above. It is assumed that oxidation took place during tryptic digestion. The observed masses of 1374.2 and 1399.9 Da appear to correspond to oxidation

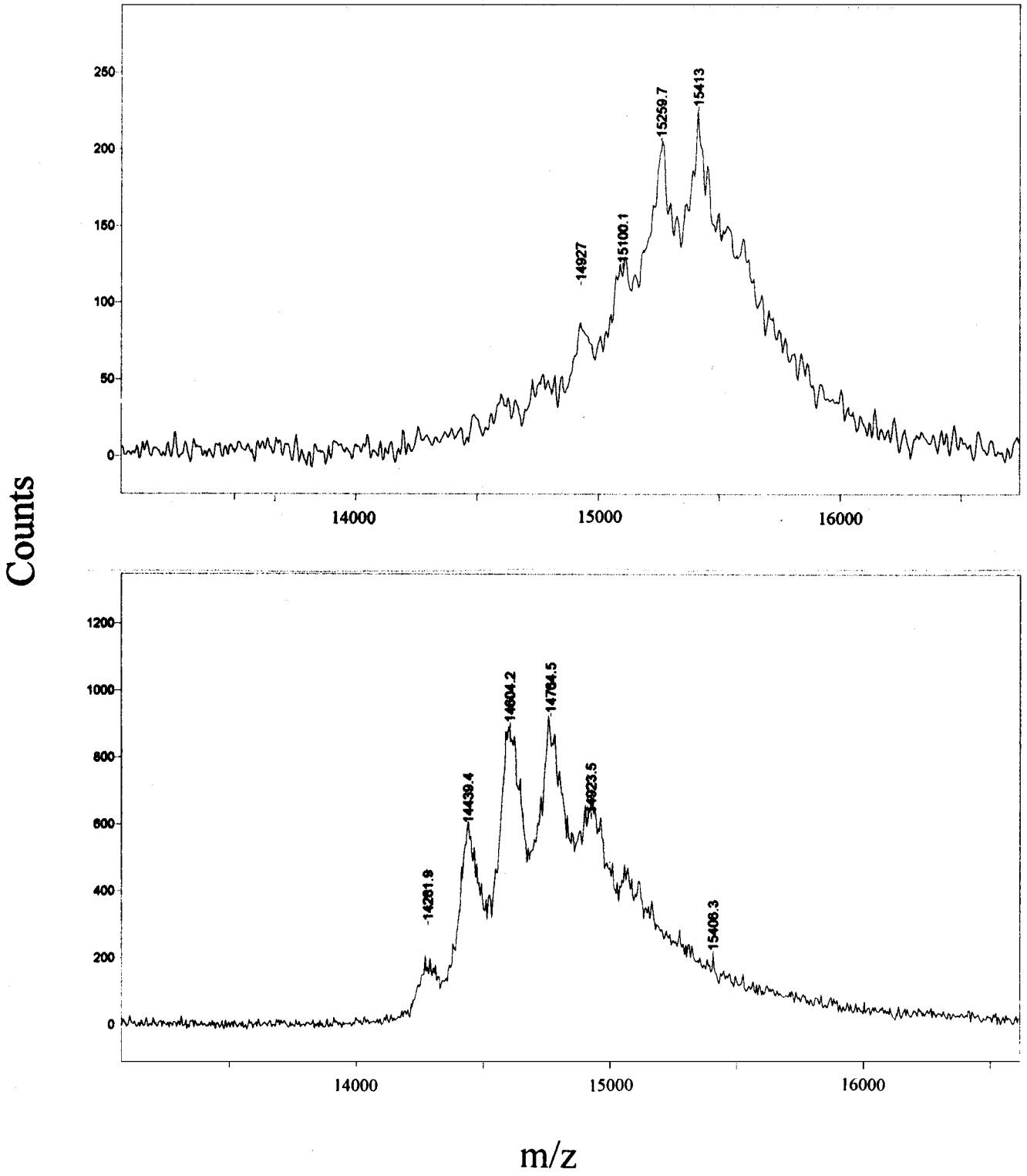

Figure 5. Mass spectrum of lysozyme glycosylated in the absence (top) and in the presence of *N*-α-acetyl-L-lysine (bottom). $(L + 6G + H)^+$ and $(L + 7G + H)^+$ are observed at 15260 and 15413 Da, respectively, as major species at top, whereas less glycosylation is observed at bottom.

products of P_{1-12}. Another major peak observed at 1161.9 Da could not be explained. It did not match the mass of any partial sequence of glucagon. It may be a contaminant.

The bottom panel of Figure 4 shows the mass spectrum obtained from tryptic digest of glucagon heated with glucose (sample C2). In addition to $(P_{13-17} + H)^+$ at 653.1 Da, $(P_{13-17} + G + H)^+$ was observed at 815.0 Da. P_{18-29} and its glucose adduct were also observed at 1510.7 and 1672.7 Da, respectively, along with their oxidation products. The ratio of glucose adducts of P_{13-17} and P_{18-29} was similar to the ratio of P_{13-17} to P_{18-29}, suggesting that the susceptibilities of these peptides toward glycosylation are similar. Neither peptide has lysine residues, and glycosylation presumably takes place at the α-amino group. Therefore, it is not surprising that they show similar proportions of glycosylated products. Interestingly, the unexplained peak also yielded a glucose adduct at 1325.5 Da, which suggests that it is also a peptide.

The only lysine-containing peptide, P_{1-12}, and its glucose adduct were not observed from sample C2 (Figure 4, bottom). It appears that, by the time P_{13-17} and P_{18-29} are glycosylated to a great extent (more than half appears as glycosylated adducts in Figure 4, bottom), the more susceptible P_{1-12} passes the initial glycosylation stage and proceeds to many different advanced reaction products. This observation is consistent with the higher susceptibility of the lysine-containing tripeptide than the nonapeptide mentioned above.

Inhibition of Glycosylation of Lysozyme (L) by N-α-Acetyl-L-lysine. To demonstrate that steps taken to minimize protein glycosylation are effective, it would be helpful to show reduced glycosylation by direct observation using mass spectrometry. Lysozyme with seven lysine residues was used for this purpose.

The MALDI-MS results from the untreated lysozyme varied from 14 290 to 14 316 Da [literature value, 14 307 Da (Canfield, 1963)] (0.1% mass accuracy as above). Small amounts of lysozyme dimers and trimers as well as $(L + 2H)^{2+}$ were also observed. The dimers and trimers are believed to be formed during the analysis.

The mass spectrum in the top panel of Figure 5 shows the heterogeneity of glycosylated lysozyme (sample D1). The two major peaks at 15 260 and 15 413 Da correspond to $(L + 6G + H)^+$ and $(L + 7G + H)^+$, respectively (expected masses, 15279 and 15441 Da). The same mass spectrum also shows smaller amounts of $(L + 4G + H)^+$ and $(L + 5G + H)^+$. Observation by MALDI-TOF-MS of progressive increases in the molecular mass by 162 Da in the glycosylation of proteins by glucose and mannose has been reported (Lapolla et al., 1994; Waddell, 1995). MALDI-TOF-MS was also applied to more complex glycoproteins and peptides (Vestal et al., 1995; Harmon et al., 1996).

The ϵ-amino groups from all seven lysine residues in lysozyme are external (Blake et al., 1965). In the mass spectrum shown in the top panel of Figure 5, where lysozyme was glycosylated with glucose only, no native lysozyme was observed and most lysozyme appeared to have been glycosylated with 4−7 glucose units, presumably at the lysine residues. Cho et al. showed that, when lysozyme is stored with glucose at 50 °C and 75% relative humidity, the content of lysine decreases most rapidly followed by that of arginine and tryptophan (Cho et al., 1984).

Cross-linking of glycosylated protein can be prevented by reacting aminoguanidine with early glycosylation products as demonstrated by Brownlee et al. (1986). They showed that the glucose-induced collagen cross-linking is greatly reduced when treated with aminoguanidine both in vivo (in diabetic rats) and in vitro (using purified collagen). Alternatively, formation of glycosylated proteins can be inhibited by free lysine or *N*-α-acetyl-L-lysine (to better mimic the ϵ-amino group of a protein), which competes with the lysine residues on the protein for glucose. From sample D2 prepared by heating lysozyme and glucose in the presence of *N*-α-acetyl-L-lysine, a MALDI-mass spectrum in the bottom panel of Figure 5 was obtained. In this case, a small amount of unmodified lysozyme was observed at 14 282 Da. Lysozyme adducts with 1−4 glucose units (14 439, 14 604, 14 765, and 14 924 Da) were major species, which suggest that addition of *N*-α-acetyl-L-lysine in the reaction mixture significantly decreased the glycosylation of lysozyme. This observation is consistent with inhibition of myosin cross-linking in freeze-dried meat by *N*-α-acetyl-L-lysine or hydrolyzed vegetable protein (Kim et al., 1984). An important difference is that in freeze-dried meat the inhibition was demonstrated as decreased cross-linking (late stage), whereas in the present case it was demonstrated as decreased glycosylation at the early stage. Since the extent of protein cross-linking is difficult to quantify, the direct measurement of the glycosylation number is believed to be a more sensitive way of demonstrating inhibition of glycosylation.

Resolution. $M/\Delta M$ under our the experimental conditions was approximately 310, which means that ΔM is 2−3 Da for the tripeptide and nonapeptide adducts. Baseline separation of oxidation products with 16 Da mass difference is possible (Figure 1). With the same resolution, ΔM for glucagon would be 11 and observation of individual oxygen adducts is still possible (Figure 3). However, ΔM is about 48 for lysozyme adducts and separation of oxygen adducts is not feasible; they appear as spikes on broad peaks of lysozyme−glucose adducts (Figure 5). Separation of lysozyme−glucose adducts with 162 mass difference is still possible when ΔM is 48. Clearly, improved resolution by delayed extraction will increase the capability of MALDI-MS for observing individual modified protein species or even different isotopic species (Vestal et al., 1995).

Quantitation. It is often stated that MALDI-TOF-MS is not a quantitative analytical technique, because of the indirect energy transfer mechanism and possible difference in desorption/ionization efficiency among different analytes. In spite of the limitations, a reasonably good quantitation might be possible. Recently, Abell and Sporns showed a good correlation between potato alkaloids determined by MALDI-TOF-MS and by HPLC (Abell and Sporns, 1996). Our discussion of different susceptibilities to glycosylation of different peptides is based on observed signal intensities and suggests that the signal intensity reasonably corresponds to the concentration of the peptide adducts.

In the present work, we were only interested in demonstrating the capability of the MALDI-MS technique for directly observing the glycosylation/oxidation products. Since glycosylation reaction involves removal of water, we carried out the reaction in the open air without humidity control to accelerate the reaction. To measure the rate constants and the activation energy of glycosylation by the MALDI-TOF-MS technique with reasonable accuracy, the peptide−glucose mixture should be heated under controlled water activity conditions.

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