Increase of Ovalbumin Glycation by the Maillard Reaction after Disruption of the Disulfide Bridge Evaluated by Liquid Chromatography and High Resolution Mass Spectrometry

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ABSTRACT: The number of glycation sites of ovalbumin was monitored by Fourier transform ion cyclotron mass spectrometry (FTICR-MS) before and after reducing the pair of the intrachain disulfide bond. Reducing the disulfide bond of the protein greatly improved the reactivity of glycation both in dry-state and solution. The glycation sites identified by MS/MS showed that the major glycation sites of the ovalbumin were lysines. Our results suggest that glycation is strongly dependent on the protein tertiary structure, with significantly stronger reaction when the protein tertiary structure is disrupted after reducing the disulfide bond. The number of glycated sites of the protein was increased from seven to twelve in dry-state and one to two in aqueous solution. The glycation sites were found to be regulated by protein tertiary structure, hydrogen bonding, and neighboring amino acid compositions.

KEYWORDS: ovalbumin, glycation, disulfide bond, mass spectrometry

INTRODUCTION

Ovalbumin, one of the only two pure proteins that can adequately meet nutritional requirements for amino acids, is the predominant protein in avian egg white, comprising approximately 54% of the total egg white protein, whose products are widely used in the food industry as a functional and nutritional ingredient.[1,2] Glycation could significantly improve the functional properties of ovalbumin, such as the emulsifying property, heat stability, gelling property, and foaming property.[3,4] Although glycation may suffer nutritional deficit due to the loss of lysine, this type of modification of proteins might still be desired, as the improved functionality of proteins can offset the detrimental loss of lysine availability or any other negative impacts.[5] Protein glycation is an early Maillard reaction that takes place when reducing sugars become attached to amino groups of proteins without the moderation of an enzyme. Several parameters, such as reaction temperature and time, properties of the protein and reducing sugar, the amino group/reducing sugar ratio and water activity (aw) could alter the rate of this nonenzymatic reaction,[6] consequently affecting the physicochemical properties of the modified proteins. A deeper characterization of Maillard reactions could lead to a better control of this process to ensure food product quality and consistency. It is essential to access and control the type and extent of this modification.

It was reported that different proteins displayed diverse glycating properties when reacted under similar conditions,[7,8] therefore protein structure plays an important role in controlling the reactivity during the glycation process. The tertiary structure of native proteins is defined by a number of

weak noncovalent interactions: hydrogen bonding, hydrophobic interactions, salt bridges, and polar interactions.[9] In addition, many proteins are also stabilized by covalent disulfide bonds. Disulfide bonds stabilize the folded state by contributing favorable enthalpy interactions in the folded states and by lowering the entropy of the unfolded states.[10,11] There are two types of disulfide bonds: intramolecular disulfide bonds occurring within a single polypeptide chain stabilize the tertiary structures of proteins while those that occur intermolecularly (i.e., between two polypeptide chains) are involved in stabilizing quaternary structure. Protein disulfide bonds can be reduced by using reducing agents such as β-mercaptoethanol, dithiothreitol (DTT) or tris (2-carboxyethyl) phosphine (TCEP) followed by blocking the liberated free sulfhydryl groups by iodoacetamide.[12] Chicken ovalbumin contains a single intramolecular disulfide bond between Cys73 and Cys120, although it has a total of six cysteines.[13] In this work, we are investigating the effect of this disulfide bond on the glycation of ovalbumin. This will provide us a better understanding of the glycation process of the proteins.

Glycation was the first step of the Maillard reaction, whose extent can be assessed by measuring the absorbance of the products to evaluate the formation of brown pigments,[14,15] the free amino groups[16] and sugar consuming[17] of the reaction system, the fluorescence intensity[18,19] and sodium dodecyl

sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) [20] of the reaction products . However, these methods do not give us a detailed glycation map of the proteins. In recent years, mass spectrometry (MS)-based techniques have provided a powerful tool in structural characterization of glycated proteins. Electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) techniques allow a detailed analysis of the nature and extent of protein modifications at a molecular level.[21] The Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) provides the highest combination of simultaneous mass measurement accuracy, resolution, and sensitivity,[22] making it an efficient tool for characterizing the structure of proteins.

In this study, we compared the glycation extent of ovalbumin under native and disulfide-bond reduced conditions in both dry-state and aqueous solution. The disulfide-bond reduced conditions were realized by reducing ovalbumin by DTT and alkylation by iodoacetamide. The glycation extent of the two samples was assessed using FTICR-MS. The glycation site of ovalbumin was identified by linear trap quadrupole-mass spectrometry/mass spectrometry (LTQ-MS/MS) to provide more detailed information for the ovalbumin glycation.

■ MATERIALS AND METHODS

Experimental Materials. Ovalbumin, glucose, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). DTT and iodoacetamide were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). All other reagents used were of analytical grade.

Sample Preparation. Ovalbumin (30 mg/mL) was prepared in 50 mM of the sodium phosphate buffer at pH 8.0 (adjusted by 0.1 M HCl). 700 μL the protein sample was mixed with 25 μL of 45 mM DTT in a centrifuge tube, incubated in a water bath at 56 °C for 15 min. Then 25 μL of iodoacetamide (100 mM) was added and incubated at room temperature for 15 min in the dark. 70 μL of 300 mg/mL glucose was added into the mixture, and the final volume was adjusted to 1000 μL by adding 180 μL of 50 mM of the sodium phosphate buffer (pH 8.0). Then the solution was split to 5 aliquots with 200 μL each. The ovalbumin-glucose disulfide-bonded sample was prepared by the same way while omitting the DTT reduction and iodoacetamide alkylation steps.

After lyophilization, the two dried mixtures were heated at 80 °C for 30 min in an oven (Thermo Fisher Scientific Inc., Waltham, MA) based on the method of Tsubokura et al.[23] with modifications. The moisture content of the lyophilized mixture was 3.17% when estimated by drying at 105 °C for 90 min under atmospheric pressure. After heat treatment, the powders were dissolved in 100 μL distilled water, and then the samples were filtered by centricon (3000 Da cutoff, Millipore, Billerica, MA) to remove the salts and free glucose and stored in a refrigerator for further use.

To prepare samples in aqueous solution, the ovalbumin and glucose mixture was prepared by simply diluting one aliquot of protein-glucose solution to make the protein final concentration to 4 mg/mL. Both forms of the solution mixture were incubated at 60 °C for 24 h. A mild heating (60 °C) was selected to limit thermal denaturation and aggregation effects. For both Maillard reactions in dry-state and aqueous solution, control experiments were carried out in the absence of glucose.

Emulsifying Properties. Only dry-state samples were used in this assay and foaming property assay since the glycation extent in dry-state was significantly higher than in aqueous solution. The emulsifying properties of the samples were measured according to Kuan et al.[24] with modifications. Briefly, 200 μL plant oil was added into 600 μL of 1 mg/mL sample solution in 50 mM sodium phosphate buffer at pH 8.0. The mixture was emulsified by a sonicator (Dismembrator Model 500, ThermoFisher Scientific, Waltham, MA) for 2 s while cooling in an ice−water bath. A 20 μL aliquot of the emulsion sample was taken from the bottom of the test tube at 0 and 10 min and immediately diluted with 200 μL of 0.1% SDS solution (prepared with 50 mM sodium phosphate buffer at pH 8.0). The absorbance of the diluted emulsion sample was then determined at 500 nm by using a nanodrop (ThermoFisher Scientific, Waltham, MA).

Foaming Properties. Foaming properties were determined by the method described by Liu et al.[25] with some modifications. Briefly, 3 mL of 2 mg/mL sample solution in a 15 mL graduated centrifugation tube was stirred by Vortex mixer (Grainger, Lake Forest, IL) at 3200 rpm for 10 min. The total sample volume was measured at 0 min for foaming capacity and 30 min for foaming stability. Foaming capacity and foaming stability were then calculated as follows:

$$\text{foaming capacity(\%)} = \frac{\text{volume after stirring} - \text{volume before stirring}}{\text{volume before stirring}}$$

$$\text{foaming stability(\%)} = \frac{\text{volume after standing} - \text{volume before stirring}}{\text{volume before stirring}}$$

Sample Digestion. Two μL (in dry-state) or 10 μL (in solution) of the protein solution was added to a 500 μL centrifuge tube containing 60 μL of 50 mM ammonium bicarbonate solution and 15 μL of 100 mM DTT. The sample was incubated at 95 °C for 5 min and then cooled in the ice bath. Twelve μL of the alkylation buffer (100 mM of iodoacetamide) was added to the tube and incubated in the dark at room temperature for 20 min. Eight μL of 0.1 μg/μL trypsin was added to hydrolyze the protein samples at 37 °C for 18 h. The reaction was then quenched by adding 2 μL of the 50% trifluoroacetic acid.

Liquid Chromatography and Mass Spectrometry Analysis. A Shimadzu HPLC, with two LC-10AD pumps, was used to generate a gradient with a 50 μL/min flow rate. Solvent A was 5% acetonitrile in H_2O, 0.1% formic acid (FA), whereas solvent B consisted of 95% acetonitrile in H_2O, 0.1% FA. For analysis of proteolytic peptides, 40 μL of digested sample was injected onto a 1.0 mm ID × 50 mm C18 column (Phenomenex Inc., Torrance, CA). After desalting for 5 min with 5% B, the peptides were eluted at 50 μL/min with a 5−10% gradient for 1 min, 10−35% for 20 min, and 30−95% for 2 min. The effluent was infused into a 12T Aglient IonSpec FTICR-MS (Agilent Inc., Santa Clara, CA). The peptides were identified by a combination of accurate masses and MS/MS. For glycated peptide identification, 30-s fractions were collected. Each fraction was subjected to a LTQ Linear Ion Trap Mass spectrometer (Thermo Fisher Scientific, Waltham, CA) for MS/MS or MS/MS/MS analysis.

To further compare the glycation extent of each peptide, the average degree of substitution per peptide molecule ovalbumin (DSP) was calculated according to the following formulation:[14]

$$\text{DSP} = \frac{\sum_{i=0}^{n} i \times I(\text{peptide} + i \times \text{glucose})}{\sum_{i=0}^{n} I(\text{peptide} + i \times \text{glucose})}$$

where I is the intensity of each ovalbumin peptide for various glycated forms, and i is the number of glucose units attached to the peptide in each glycated form.

■ RESULTS AND DISCUSSION

Functional Properties. As shown in Figure 1, the emulsifying activity of the reductive-alkylated (OVA-DTT) and the glycated (OVA-G) ovalbumin was increased than that of the native protein (OVA). When the reductive-alkylation and glycation were combined (OVA-G-DTT), the protein showed the highest emulsifying activity. DTT is able to reduce the disulfide bond of proteins, changing protein conformations with exposed hydrophobic domains, therefore, increasing emulsifying activity of proteins. Poon et al.[26] reported that the DTT reduction improved the emulsifying activity of
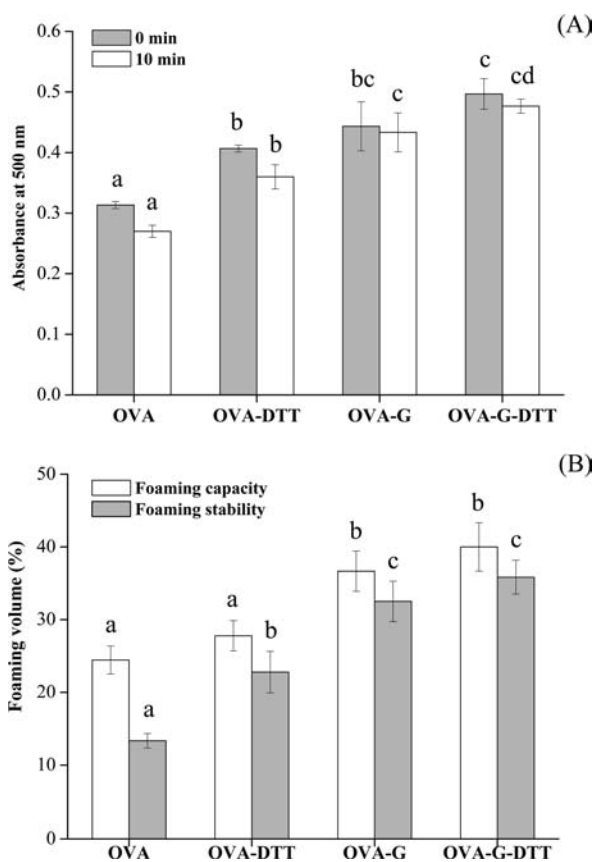
Figure 1. Emulsifying properties (A) and foaming properties (B) of samples: OVA: native ovalbumin; OVA-DTT: ovalbumin reduced by DTT; OVA-G: ovalbumin glycated with glucose; OVA-G-DTT: ovalbumin glycated after reductive-alkylation. The letters (a–d) on the tops of columns indicate significant ($p < 0.05$) differences among samples.

lysozyme. Alkylation can also improve the emulsifying property of proteins.[27] In addition, glycation has been widely used to improve the emulsifying activity of proteins.[8] Therefore, after ovalbumin is treated with reductive-alkylation and glycation, its emulsifying activity was significantly better than its original form.

The foaming properties of the ovalbumin exhibited similar trends of enhancement after all three modifications. The combination of reductive-alkylation and glycation of ovalbumin gained the best foaming capacity and stability. When the intramolecular disulfide bonds are reduced in proteins, the molecular flexibility and surface hydrophobicity can be increased, consequently, improving the foam forming and stabilizing capacities.[28,29] Similar to our results, Haar et al.[30] found a higher foaming stability in α-lactalbumin resulting from the elevated glycation extent.

The Glycation Extent. It should be noted that both of the lysyl and arginyl are potential glycated sites and it has been reported that once a site is linked with the reducing sugar, trypsin is not functional on this site any more.[31,32] We first performed a MS survey scan for the glycated samples using pepsin digestion. The results showed that after 30 min of Maillard reaction, the unglycated peptides and glycated peptides coexisted in the sample solution as shown in Figure 2. The m/z peaks of 732.4159^{3+}, 786.4338^{3+}, and 840.4517^{3+} were identified as the unglycated peptides 41−59, its singly glycated form, and doubly glycated form, respectively. Similarly, the m/z peaks of 676.4417 and 838.4946 represented the unglycated peptides 178−183 and its singly glycated form. This partial glycation suggested that trypsin can be used as the protease to identify the glycated peptides. After digestion, each glycated peptide will generate a miss-cleaved tryptic peptide. Figure 3 showed FTICR mass spectra of the peptides and their corresponding glycated forms of the native ovalbumin. The glycated forms of the peptides can be easily determined from the mass difference induced by glycation.[33,34] Theoretically, if a peptide was monoglycated by glucose, the corresponding m/z peaks with 1, 2, 3, or 4 charges will display a mass increase of 162.0528 Da, with m/z change of 162.0528, 81.0264, 54.0176, and 40.5132, respectively. For dual-glycated peptides, the mass increase will be equal to 324.1056 Da. For example, the m/z peaks of the unglycated peptides 187−199, 200−218, 20−50, and 85−104 (Figure 3) were 778.3640^{2+}, 762.0557^{3+}, 873.9369^{4+}, and 761.0662^{3+}, while the corresponding m/z of glycated peptides were 859.3902^{2+}, 816.0734^{3+}, 914.4504^{4+}, and 815.0836^{3+}, respectively. The m/z differences of these peaks were 81.0262, 54.0177, 40.5135, and 54.0174 Da, respectively, indicating that all of these peptides were modified by one
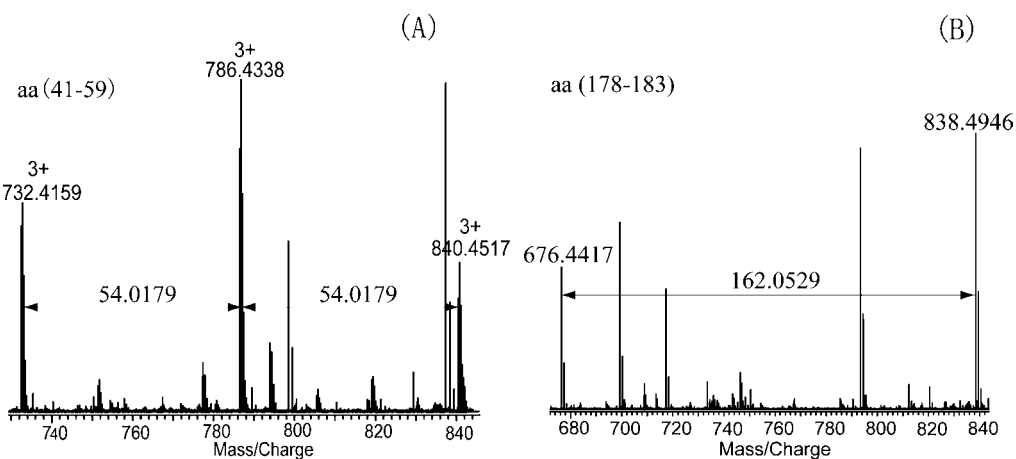

Figure 2. The FTICR-MS spectra of representative glycated peptides of disulfide-reduced ovalbumin after pepsin digestion. A. Peptides 41−59 at m/z 732.4159^{3+}, B. peptides 178−183 at m/z 676.4417. The identified peptides are labeled using residue numbers. Glycation is indicated by double-headed arrows. The m/z difference of between the glycated and unglycated peptides are indicated above the arrows.
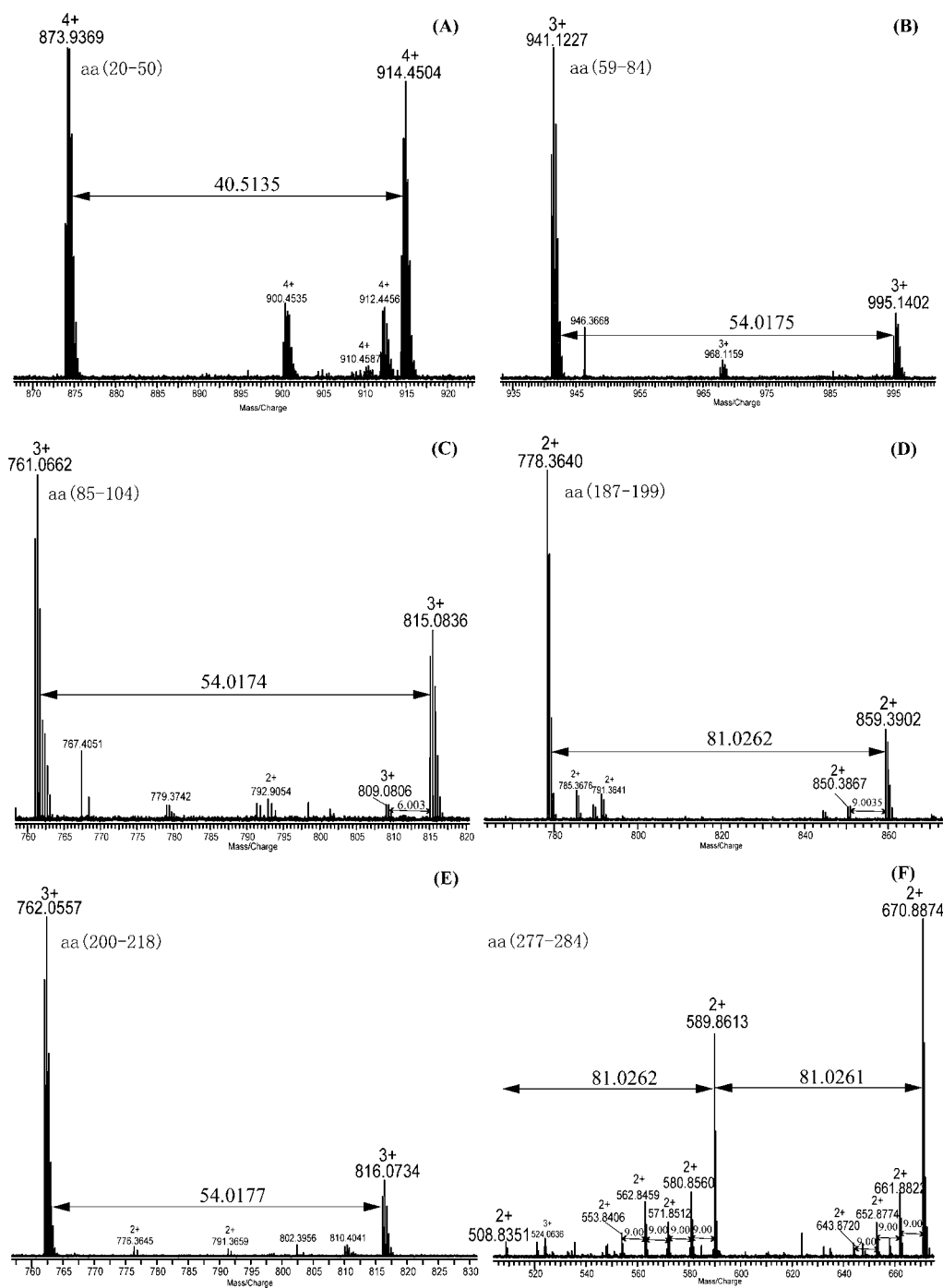
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Figure 3. The FTICR-MS spectra of the glycated peptides of the disulfide-bonded ovalbumin. (A) Peptides 20−50 at m/z 873.9369^{4+}, (B) peptides 59−84 at m/z 941.1227^{3+}, (C) peptides 85−104 at m/z 761.0662^{3+}, (D) peptides 187−199 at m/z 778.3640^{2+}, (E) peptides 200−218 at m/z 762.0557^{3+}, and (F) peptides 277−284 at m/z 508.8351^{2+}. The identified peptides are labeled using residue numbers. Glycation is indicated by double-headed arrows. The m/z difference of between the glycated and unglycated peptides are indicated above the arrows.

molecule of glucose. The ion peaks with m/z of 589.8613^{2+} and 670.8874^{2+} (Figure 3F), were found to have a mass shift of 162.0524 and 324.1046 Da, respectively, from the peptides 277−284 with m/z of 508.8351^{2+}, indicating that two molecules of glucose were attached to the peptides 277−284. In the mass spectrum of the ion peaks 589.8613^{2+} and 670.8874^{2+}, a series of negative mass shift of 18.0106, 36.0212, 54.0308, and 72.0414 Da were observed (Figure 3F). These neutral losses are the dehydration products of the two glycated peptides with consecutive water losses ranging from one to

four. Similarly, the ion peak with m/z of 809.0806^{3+} was the dehydration product of the glycated form of the peptides 85−104 with m/z of 761.0662^{3+} (Figure 3C).

Tables 1 and 2 list all of the peptides that are glycated without and with DTT treatment in dry-state and in solution, respectively. When the protein was not disturbed by DTT, only six peptides, including peptides 20−50, 59−84, 85−104, 187−199, 200−218, and 277−284 were modified with glucose. After DTT disrupted the disulfide bonds between Cys73 and Cys120, overall eleven peptides were glycated. In addition to

Table 1. Comparison of the Glycated Peptides with 30 min Heating at 80 °C in Dry-State before and after DTT Reduction

native peptide	position	error (ppm)	sequence	modified peptide	mass shift	DSP	site	B-factor (Å²)
OVA-G				OVA-G				
873.9369⁴⁺	20−50	0.937	VHHANENIFYC (CAMᵃ)PIAIMSAL AMVYLGAKDSTR	914.4504⁴⁺	162.054	0.47	K46	44.16
941.1227³⁺	59−84	−1.35	FDKLPGFGDSIEA QC(CAM) GTSVNV HSSLR	995.1402³⁺	162.0525	0.17	K61	57.31
761.0662³⁺	85−104	0.771	DILNQITKPNDVY SFSLASR	809.0806³⁺/ 815.0836³⁺	144.0432/162.0522	0.36	K92	54.03
778.3640²⁺	187−199	−0.148	AFKDEDTQAMPFR	850.3867²⁺/ 859.3902²⁺	162.0524	0.21	K189	48.15
762.0557³⁺	200−218	2.68	VTEQESKPVQMM YQIGLFR	816.0734³⁺	162.0531	0.18	K206	71.36
508.8351²⁺	277−284	1.78	KIKVYLPR	580.8560²⁺/ 589.8613²⁺	144.0418/162.0524	0.93	K277	53.84
				661.8822²⁺/ 670.8874²⁺	306.0942/324.1046	1.55	K277,K279	53.84/50.51
OVA-G-DTT				OVA-G-DTT				
873.9361⁴⁺	20−50	0.0208	VHHANENIFYC (CAM)PIAIMSAL AMVYLGAKDSTR	914.4493⁴⁺	162.0528	0.62	K46	44.16
708.8990²⁺	47−58	−0.302	DSTRTQINKVVR	789.9249²⁺	162.0518	0.31	K55/R50	48.32/37.47
941.1232³⁺	59−84	−0.823	FDKLPGFGDSIEA QC(CAM) GTSVNV HSSLR	995.1410³⁺	162.0534	0.19	K61	57.31
761.0667 ³⁺	85−104	1.43	DILNQITKPNDVY SFSLASR	815.0837 ³⁺	162.0510	0.35	K92	54.03
949.1557³⁺	105−126	−2.38	LYAEERYPILPEYL QC(CAM) VKELYR	1003.1721³⁺	162.0492	0.46	K122	55.68
1025.2168³⁺	159−186	−1.05	NVLQPSSVDSQTA MVLVNAIVFKGL WEK	1079.2342³⁺	162.0522	0.15	K181	32.95
778.3632²⁺	187−199	−1.18	AFKDEDTQAMPFR	850.3824²⁺/ 859.3888²⁺	144.0384/162.0512	0.23	K189	48.15
762.0554³⁺	200−218	2.28	VTEQESKPVQMMYQIGLFR	816.0727³⁺	162.0519	0.28	K206	71.36
541.2732²⁺	219−228	1.01	VASMASEKMK	613.2946²⁺/ 622.2993²⁺	144.0428/162.0522	0.44	K226	65.99
508.8353²⁺	277−284	1.78	KIKVYLPR	580.8561²⁺/ 589.8612²⁺	144.0416/162.0518	0.93	K277	53.84
				661.8817²⁺/ 670.8873²⁺	306.0928/324.104	1.35	K277, K279	53.84,50.51
858.7860³⁺	360−381	−0.149	ADHPFLFC(CAM) IKHIATNAVLFFGR	912.8034³⁺	162.0522	0.64	K369	27.71

ᵃCAM refers to carbamidomethy.

Table 2. Comparison of the Glycated Peptides with 24 h Heating at 60 °C in Aqueous Solution before and after DTT Reduction

native peptide	position	error (ppm)	sequence	modified peptide	mass shift	DSP	site	B-factor (Å²)
OVA-G				OVA-G				
778.3636²⁺	187−199	−0.662	AFKDEDTQAMPFR	859.3901²⁺	162.0530	0.071	K189	48.15
OVA-G-DTT				OVA-G-DTT				
778.3641²⁺	187−199	−0.0179	AFKDEDTQAMPFR	859.3900²⁺	162.0518	0.095	K189	48.15
762.0542³⁺	200−218	0.705	VTEQESKPVQMMYQIGLFR	816.0720³⁺	162.0534	0.064	K206	71.36

the six peptides glycated under the native conditions, five more peptides were attached with the glucose, including the peptides 47−58, 105−126, 159−186, 219−228, and 360−381 (Figure 4). Compared with the dry-state, much less glycated peptides were detected in the aqueous Maillard reaction. Only one glycated peptide was found in the native ovalbumin-glucose after 24 h heating in solution, while one additional glycated peptide was observed when the disulfide bond was reduced in aqueous solution. The improved glycation extent of ovalbumin suggests that the reducing process has altered the protein conformation, enabling an increased solvent accessibility to the protein. Given the increased solvent accessibility, the reducing sugar, glucose had much more chance to react with exposed glycation sites, leading to an elevated degree of glycation.

Takahashi et al. compared the conformation and stability of the disulfide-bonded ovalbumin with those disulfide-reduced form and they found that ovalbumin remained a native-like

conformation in its disulfide-reduced form, however the local conformation of the reduced form fluctuated more than that with disulfide-bonded one.[35] This is consistent with our mass spectrometry data presented here. After removing the disulfide bond, ovalbumin exhibited 70% more glycated sites (5 more) than disulfide-bonded form. In Table 1, it can be seen that the peptide containing K122 was reactive with the sugar in the disulfide-reduced form, while not in the disulfide-bonded form. The increased activity of glycation in the vicinity of the disulfide bond suggests that a more flexible conformation was generated after reducing the disulfide bond. Similarly, the neighboring amino acid K55 (or R50) in peptides 47−58 gained the glycation reactivity after disulfide bond was reduced. It should be noted that there are two possible glycation sites on this peptide. We were not able to determine the exact glycation site of this peptide due to the limited MS/MS sensitivity.
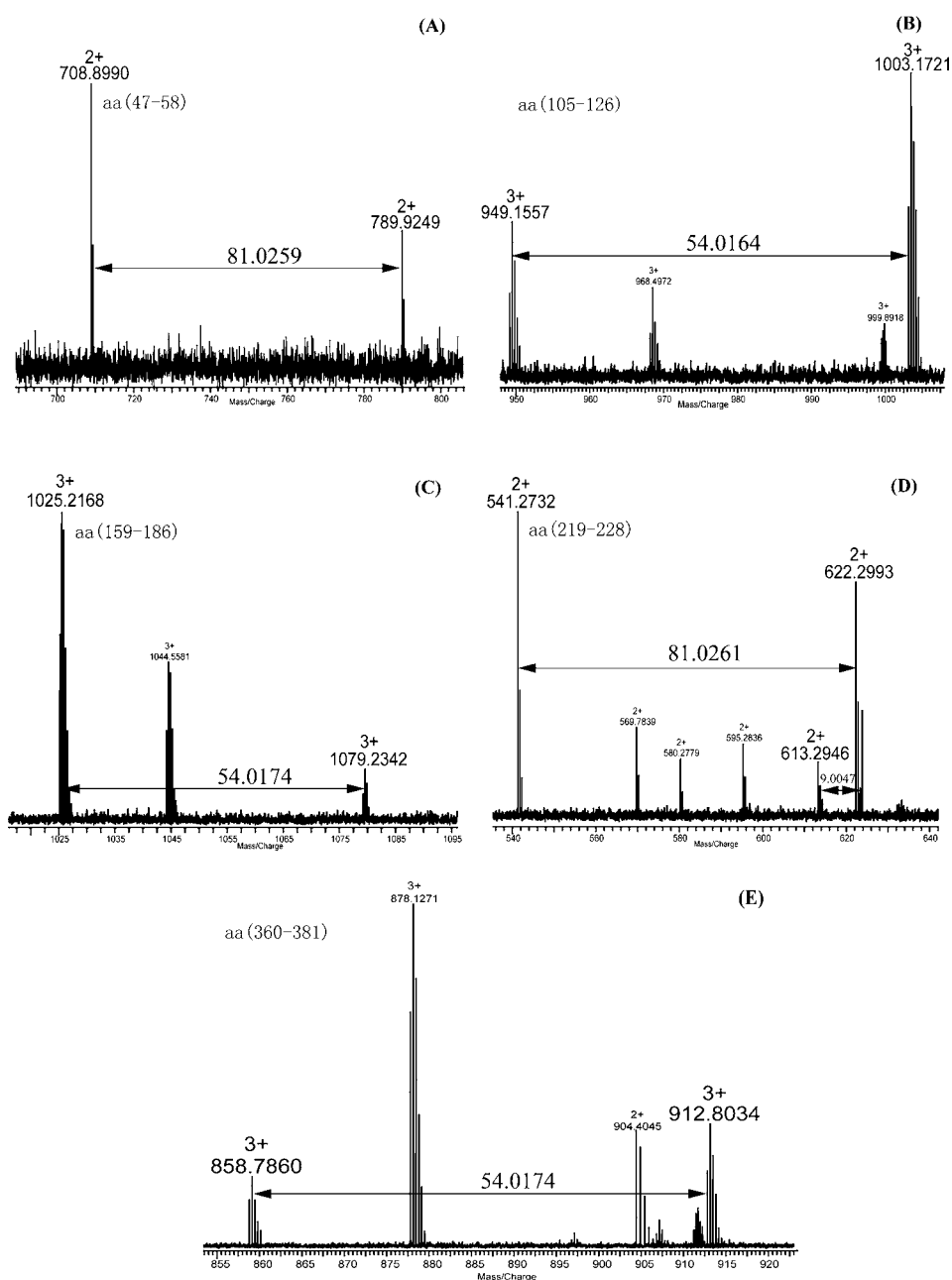
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Figure 4. The FTICR-MS spectra of the additional glycated peptides of the disulfide-reduced ovalbumin. (A) Peptides 47−58 at m/z 708.8990 [2+], (B) peptides 105−126 at m/z 949.1557[3+], (C) peptides 159−186 at m/z 1025.2168[3+], (D) peptides 219−228 at m/z 541.2732[2+], and (E) peptides 360−381 at m/z 858.7860[3+]. The identified peptides are labeled using residue numbers. Glycation is indicated by double-headed arrows. The m/z difference of between the glycated and unglycated peptides are indicated above the arrows.

To further understand the relative glycation reactivity of the peptides, DSP of each glycated peptide was calculated. The DSP results of the glycated peptide in dry-state and aqueous solution were presented in Tables 1 and 2, respectively. The DSP of the same peptide glycated in dry-state and in aqueous solution showed drastic difference, with a much higher extent in dry-state than in aqueous solution. For example, when the peptides 187−199 of the native ovalbumin was glycated in dry state, the DSP was 0.21, whereas the DSP of 0.071 was evaluated in aqueous solution, indicating that the glycation was much more promoted in dry-state compared to in solution Maillard reaction. In dry-state, the reactant concentration is much higher and energy transfer is much more efficient, therefore, the reactions are faster than their liquid-phase

counterparts. In addition, a substantial loss of energy to the solvent in the liquid-phase reactions causes a decreased reactivity.[36][21] Among all of the glycated peptides, peptides 277−284 presented the highest DSP value, implying that this peptide contained the most active glycation site in ovalbumin. Compared to the native protein, the DSP of the reduced protein exhibited a little higher value in most peptides, i.e., peptides 20−50, 59−84, 187−199, and 200−218 all showed enhanced glycation extent.

The Glycation Sites of Ovalbumin. To better understand the structure of the glycated ovalbumin, the sites of the glycation should be identified. During the Maillard reaction process, the functional groups that could be preferably glycated are the α-amino group of the N-terminus, the ε-amino groups
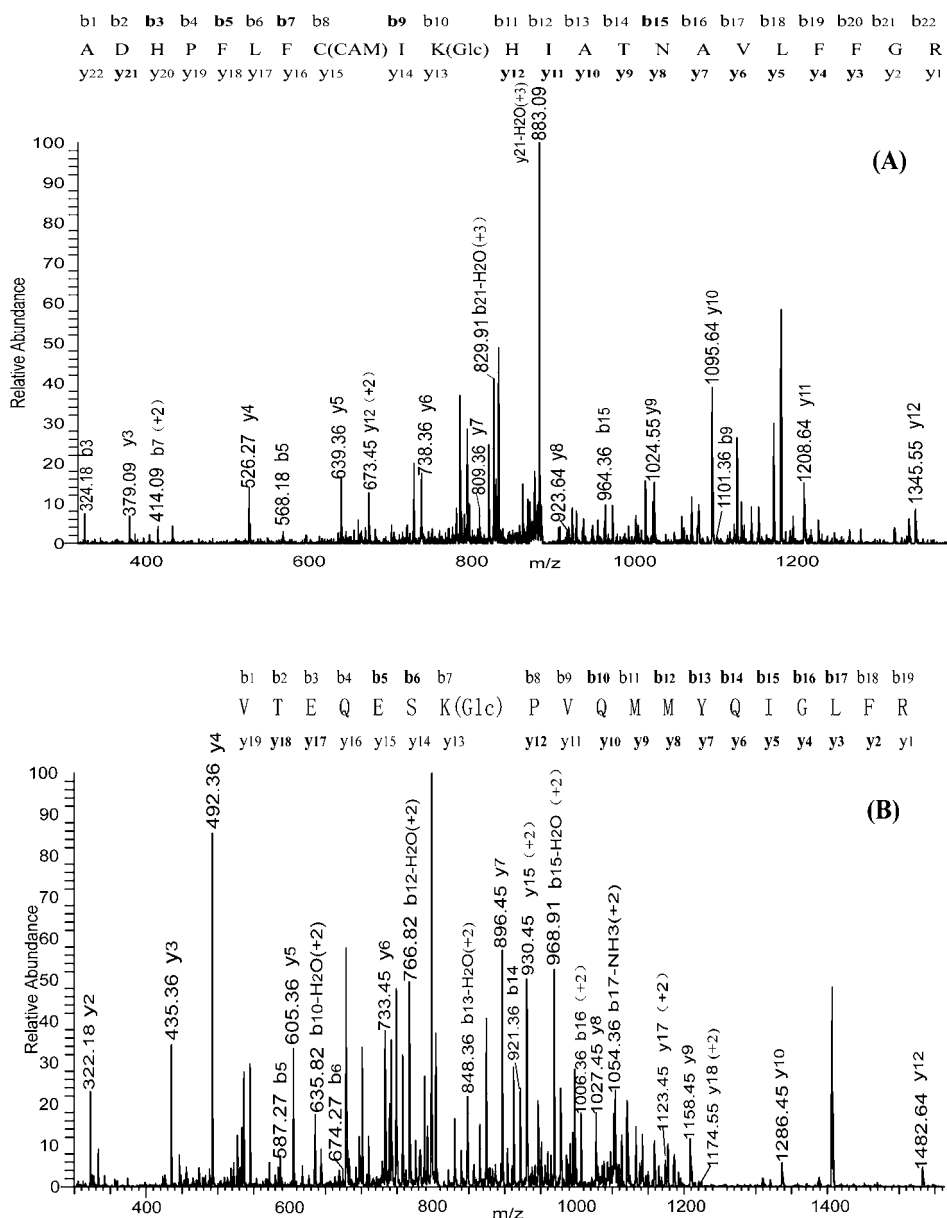
Figure 5. The CID MS/MS of the glycated peptides. (A) The glycated peptides 360−381 (ADHPFLFC(CAM)IKHIATNAVLFFGR) at m/z 912.8034^{3+} and (B) the glycated peptides 200−218 (VTEQESKPVQMMYQIGLFR) at m/z 816.0727^{3+}. The sequence of the each peptide is shown on top of the spectrum. Mainly, b and y ions are shown in the mass spectra. CAM and Glc refer to carbamidomethyl and glucose, respectively.

of the lysine residues and the guanidine groups of the arginine residues in lysozyme.[37] Figure 5A is the CID mass spectrum of the glycated peptides 360−381 with the sequence of ADHPFLFC(CAM)IKHIATNAVLFFGR at m/z 912.8034^{3+} (CAM refers to carbamidomethyl). The accurate mass and tandem mass spectrum of the peptide clearly confirmed the sequence and the glycation site was determined at K369. It should be noted that in this peptide, the C-terminal peptide R is excluded from being a glycation site simply because it is a tryptic peptide. As mentioned previously, the tryptic truncation sites can only be those not glycated. Figure 5B shows the mass spectrum of the fragmentation of glycated peptides at m/z of 816.0727^{3+}. A number of y-series ions and b-series ions in the figure matched well with the fragmentation of peptide VTEQESK(Glc) PVQMMYQIGLFR, confirming that the glucose was linked to K206. Similarly, the glycated positions for peptides with m/z of 859.3902^{2+}, 815.0837^{3+}, and

622.2993^{2+} (Figure 6) were determined to be K189, K92, and K226, respectively.

For lysines, it has been suggested that the properties of neighboring amino acids play a critical role in determining whether a given lysine is glycated or not. Both positively and negatively charged amino acids can catalyze glycation of a lysine if they are located close to the lysine. For example, the presence of a histidine or lysine residue close to a lysine in either the primary or the tertiary structure has been reported to promote the glycation of lysines.[38−41] It has also been suggested that the acidic amino acids close to lysines in either the primary or the tertiary structure catalyze the Maillard reaction. In addition, model studies have actually shown that aspartate residues situated in the vicinity of a lysine can enhance the glycation of that lysine. The same appears to be the case for arginines situated proximal in the tertiary structure to lysines.[42]
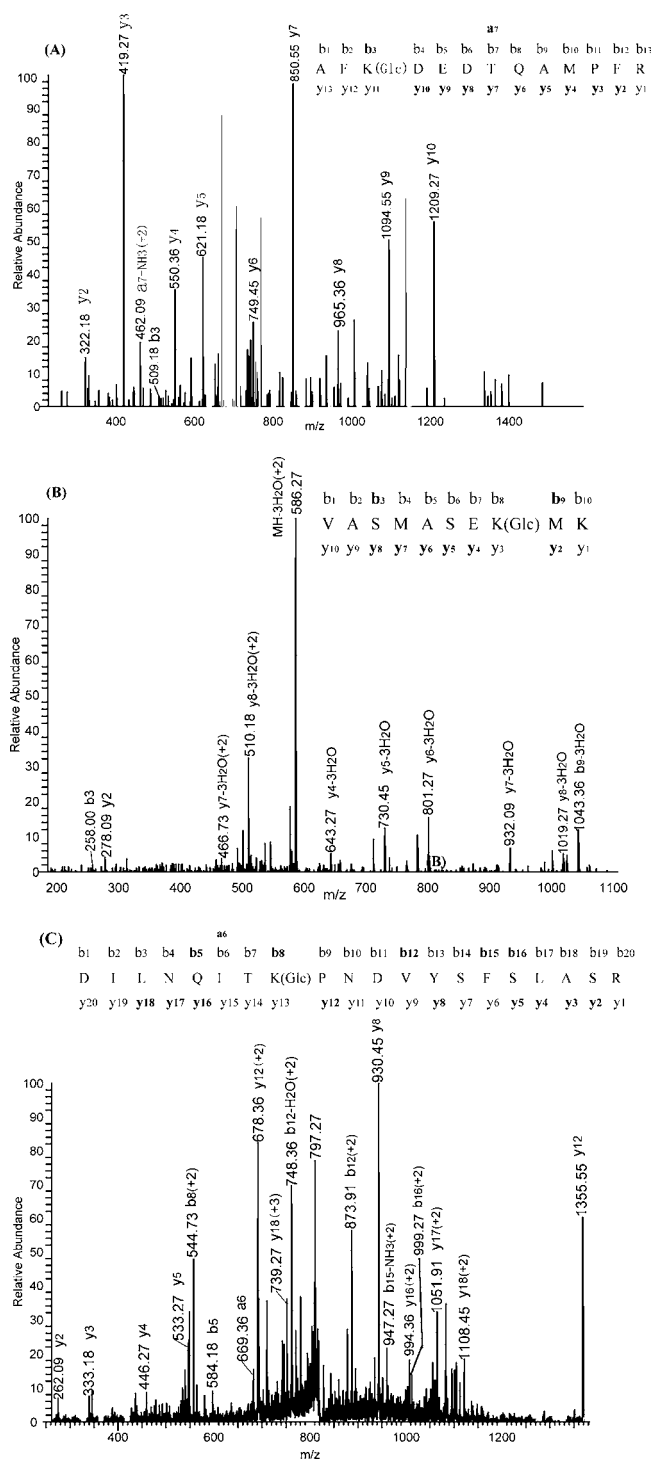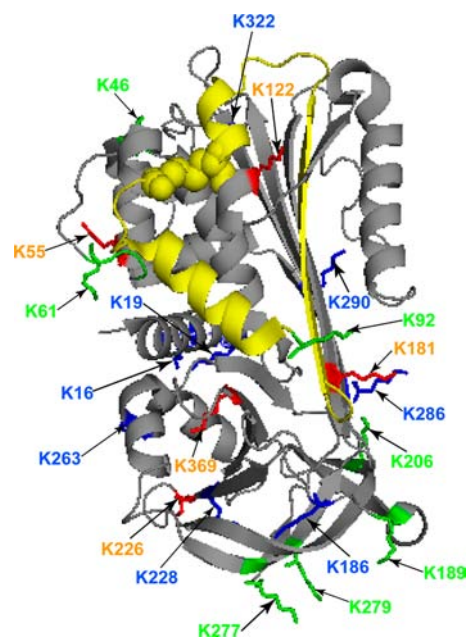
Figure 7. Ribbon diagram of the glycated ovalbumin (PDB IJTI). The lysines are color coded as follows: gray = framework of ovalbumin; green = glycated amino acid residues of the native ovalbumin; red = extra glycated amino acid residues of the reduced ovalbumin; and blue = unglycated lysine residues. The disulfide bond is shown in spheres. The peptides (73−120) containing the disulfide bond are shown in yellow.

Figure 6. The CID MS/MS of the glycated peptides. (A) The glycated peptides 187−189 (AFKDEDTQAMPFR) at m/z 859.3888^{2+}, (B) the glycated peptides 219−228 (VASMASEKMK) at m/z 622.2993^{2+}, and (C) the glycated peptides 85−104 (DILNQITKPNDVYSFSLASR) at m/z 815.0836^{3+}. The sequence of the each peptide is shown on top of the spectrum. Mainly, b and y ions are shown in the mass spectra. Glc refers to glucose.

reacted with glucose. In addition, the microenvironment around the residues also made contribution to the reactivity. K277 and K279 were within the sequence of EERKIKVYLPR (aa274−284), in which there were two acidic glutamic acid residues and two basic arginine residues close to the lysines. They could function as catalysts for the glycation of the lysine. Also, the two lysine residues which were basic could catalyze each other during glycation process. Similarly, other glycated sites of the native ovalbumin, K46, K92, K61, K189, and K206 all exhibited relatively high B-factor values (Table 1). It is known that B-factors of protein crystal structures reflect the fluctuations of atoms around their average positions. Higher B-factor values indicate flexible residues, while lower B-factor values indicate rigid residues.[43,44] Given the high flexibility, the lysine residues with higher B-factor values have higher possibility to react with glucose. In contrast, the lysine residues with lower B-factor values, i.e., K181 (32.95 Å^2) and K369 (27.71 Å^2), were not linked to glucose in its native conditions.

The B-factor of K226 is 65.99 Å^2, as high as those of glycated lysines, however, it was not glycated until the disulfide was reduced. A closer look at the structure of the protein reveals that this amino acid is involved in multiple hydrogen bonds. Three pairs of hydrogen bonds are formed between this lysine and three other amino acid residues, Met222, Asp247, and Glu248. The glycation was most likely prohibited by these hydrogen bonds. Similarly, the other lysine residue with high B-factor, K122 was not glycated probably because it forms two hydrogen bonds with Leu118 and Val121. In comparison, the glycated lysines in native form of ovalbumin are less involved in hydrogen bonding. For examples, K189 and K92 do not form hydrogen bond with other amino acids, while K277 forms only one hydrogen bond. K206 is the lysine residue with highest B-factor, however, its glycation degree was lower than others

In this study, the residues of K277 and K279 were found to be the most reactive glycation sites as judged by the DSP data. From the tertiary structure of ovalbumin (Figure 7), it can be seen that K277 and K279 were most exposed to the solvent with B-factor values above 50 Å^2, making them ready to be

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(DSP = 0.18). This can also attributed to the inhibition from the hydrogen bonds that it formed with three other amino acid residues, Phe198, Ser384, and Pro385.

In the reduced form of ovalbumin, K122 and K55/R50 located in the α-helix near the disulfide bond area were glycated. This is most likely attributed to the local conformational change after reducing the disulfide bond. Interestingly, K369, which was previously unglycated exhibited very high reactivity for glycation (DSP = 0.64). This gain of reactivity suggests that disruption of disulfide bond not only introduced a conformational change in the vicinity of the disulfide bond, but also in a distal site. These lysine residues obtained more chance to react with glucose after the conformational change induced by disulfide-bond reduction, resulting in an increased DSP value. The other factor, the catalytic basic residue H370 may also play a role here to promote the glycation of K369. Similarly, K226 and K181 also gained enhanced reactivity of glycation due to the distal conformational changes.

Overall, 12 of the 20 lysine residues of the ovalbumin have been found to be glycated. The rest of the lysine residues including K16, K19, K186, K228, K263, K286, K290, and K322 were not glycated despite of the existence of some acidic or basic residues in the close vicinity under our experimental conditions. It should be noted that due to the limited glycation of some of the lysines, the glycated form of the peptide may be escaped for detection. In particular, trypsin digestion of glycated peptides many times will generate large peptides because glycation can block the tryptic cleavage at the modified sites. For example, the smallest tryptic peptide containing K263 is peptide Ile^{229}-R^{276} with a mass of 5425.71. The glycated form of this peptide may not be detected when it is minimally glycated. In order to confirm this, we performed the pepsin digestion of the glycated ovalbumin under the same heating conditions, and peptide containing K263 was captured at a smaller mass range (data not shown). Overall, we detect almost all of the peptides containing lysine residues except the peptide containing K322 under our experimental conditions. The smallest peptide containing K322 is Y^{291}−K^{322} with a mass of 3293.63. Amide nitrogen of Asn292 can be modified by heterogeneous carbohydrate chains.[45] At least six different peptides have been identified to be glycosylated on this site.[46−48] The peptide containing K322 was missed in our LC/MS analysis after trypsin digestion most likely due to this heterogeneity. Nevertheless, our direct comparison between the disulfide-reduced form and disulfide-bonded form of ovalbumin still provides sufficient information to illustrate the improvement of glycation after reducing the disulfide bond.

Ovalbumin is known to form fibrillar types of aggregates upon aggregation and it can form a gel at high protein concentrations.[49] The aggregation of proteins is typically induced by heat, enzymatic cleavage, or other processes that affect the protein conformations. It was reported that the half-time for ovalbumin to be denatured and aggregated was less than 2 min at 80 °C in the aqueous solution, while at 68.5 °C, it was about 6 h.[50] In this work, we are investigating the effect of the disulfide bond on the Maillard reaction, which involves heating and breaking the disulfide bond. Care must be taken not to introduce the irreversible aggregation of the protein, so that mass spectrometry analysis can be performed on the heat treated samples. We selected heating at 80 °C for 30 min in dry-state and 60 °C for 24 h in the aqueous state since the Maillard reaction was fast, and there was no apparent aggregation under these two conditions. By reducing the

disulfide bond of the protein, the number of glycated peptides was increased from six to eleven in dry-state and one to two in aqueous solution. The glycation sites were found be regulated by several factors, including the protein tertiary structure, hydrogen bonding, and neighboring amino acid compositions. Conformational changes induced by reducing disulfide bonding can drastically affect the glycation process.

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Notes
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