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Effect of Fructose and Glucose on Glycation of β-Lactoglobulin in an Intermediate-Moisture Food Model System: Analysis by Liquid Chromatography−Mass Spectrometry (LC−MS) and Data-Independent Acquisition LC−MS (LC−MS^E)

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Supporting Information

ABSTRACT: To evaluate the effect of glucose and fructose on the glycation of β-lactoglobulin (β-Lg) in intermediate-moisture food (IMF), model systems consisting of β-Lg, glucose/fructose/sorbitol, glycerol, and water were established. All systems were stored at 25 and 35 °C for 2 months. The progress of the Maillard reaction and the mass change of β-Lg were investigated by the browning assay and gel electrophoresis, respectively. Meanwhile, liquid chromatography−mass spectrometry (LC−MS) and data-independent acquisition LC−MS (LC−MS^E) were used to monitor the glycation extent and the glycated sites of β-Lg. The results indicated that glucose had a higher reaction activity of glycation than fructose, but both sugars had similar preference on the glycation site for β-Lg. The ranking order from high to low for the 9 detected glycated sites was L 1, K 91 > K 47 > K 70, K 77, K 83, K 100 > K 75 > K 135 for both sugars.

KEYWORDS: *Glycation, intermediate-moisture food, reducing sugar, lactoglobulin, LC−MS, LC−MS^E*

INTRODUCTION

Intermediate-moisture food (IMF), such as nutritional bars, refers to food products with water activity (a_W) in the range of 0.5−0.9.[1] The formula of IMF often contains a high portion of proteins and reducing sugars. Whey protein is an important protein source for IMF, which mainly contains β-lactoglobulin (β-Lg), α-lactalbumin, bovine serum albumin (BSA), and immunoglobulin. β-Lg is the most abundant protein fraction in whey proteins, and it has two variants: A (18 363 Da) and B (18 277 Da), each containing 162 amino acids. The two variants only differ in two amine components: Asp 64 and Val 118 in variant A and Gly 64 and Ala 118 in variant B.[2] In addition, corn syrup and high-fructose corn syrup are often used in IMF to serve as sweeteners, humectants, and plasticizer to provide the appropriate taste and texture. Their major components, fructose and glucose, are both reducing sugars. Because of the high portion of proteins and reducing sugars in the formula, the Maillard reaction often occurs, which is a series of chemical reactions between amino groups of amino acids, peptides, or proteins and carbonyl groups of reducing sugars, starting from the glycation of protein, proceeding to the formation of sugar-derived protein adducts and cross-links known as advanced glycation end-products (AGEs), and eventually producing brown and water-insoluble compounds named melanoidins.[3] In the early stage of the Maillard reaction, also known as glycation, free amino group and carbonyl group are condensed to form a Schiff base. The Schiff base then undergoes irreversible rearrangement to form Amadori or Henys rearrangement depending upon whether the reducing sugar is an aldose or ketose. The glycation activity of aldose and ketose had been studied, and most studies agreed that aldose is more reactive than ketose in glycation.[4−9] However, most of these studies focused on glycation extent, and few explored the differences on glycation sites regarding aldose and ketose.

For IMF, the Maillard reaction would result in changes of color, texture, flavor, and nutritional value during storage, causing detrimental effects on the product quality and consumer acceptance. The study of the Maillard reaction, especially glycation, in IMF might provide a theoretical basis on preventing quality loss. However, few studies investigated the glycation of proteins in IMF. According to previous studies on protein glycation in solution, milk powders, and dry state, the glycation would be affected by reaction conditions, including water activity and reaction medium.[10] Therefore, it would be meaningful to reveal the glycation extent and sites of proteins in IMF.

To monitor the Maillard reaction, a variety of methods have been established on the basis of various compounds formed during the Maillard reaction. The browning assay[11] is developed to evaluate the formation of brown pigments, while *O*-phthaldialdehyde (OPA)[4,12−15] and fluorescamine[16,17] assays are used to measure the free amino groups. AGEs can be detected by fluorescence measurements,[4,5] and the furosine assay[14] measures furosine originating from acid hydrolysis of glycated lysyl residues. In comparison to the regular analytical

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methods, the application of mass spectrometry (MS) would give more accurate and detailed information regarding the protein glycation. The development of soft ionization technologies, such as fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI), enables the analysis of proteins and peptides, thus providing a possibility for monitoring and evaluating the glycation number of adducted sugars and glycated sites of proteins. In previous studies, the glycation extent of β-Lg had been extensively determined by liquid chromatography−electrospray ionization−mass spectrometry (LC−ESI−MS)[14,18−23] and MALDI−MS.[4,5] The glycated sites are analyzed by MS (MALDI−MS[23−27] and ESI−MS[20,27]) and tandem mass spectrometry (MS/MS) techniques, which had been reviewed by Oliver.[10] MS techniques could reveal the possible glycated sites of proteins, but they lack the ability to determine the accurate glycated amino residues, which can be overcome by MS/MS through generation of a parent ion and a fragmental ion to facilitate the location of the modified sites. However, the MS/MS approach is usually adopting a data-dependent acquisition (DDA) method, which generates a fragment ion based on the intensity of the parent ion. Therefore, this method is costly for time and has a lack of reproducibility.[28] Even though there are other methods, such as collision-induced dissociation (CID),[20,29,30] the fragmentations produced more or less relied on the parent ions. Besides MS and MS/MS, there is another comparatively novel technique named LC−MS^E, which is a parallel acquisition approach, generating parent ions by low energy and fragment ions by elevated energy throughout the whole chromatographic profile; therefore, it is possible to correlate the MS and MS^E values to provide detailed information for all of the peptides.[31]

The purpose of this study was to evaluate the difference between glucose and fructose in the glycation activity and site preference with β-Lg in IMF. IMF model systems containing β-Lg, glucose/fructose/sorbitol, glycerol, and water were established. Sorbitol was used as a control because of its nonreducing property, high solubility in water (220 g/100 g of water at 20 °C), and a molecular weight of 182 Da adjacent to fructose and glucose. Glycerol was a common humectant/plasticizer for maintaining texture and lowering the water activity.[1] The effect of fructose and glucose on β-Lg during storage of IMF systems was evaluated by analyzing the glycation extent and glycation sites of the two sugars on β-Lg using LC−ESI−MS and LC−ESI−MS^E, respectively.

MATERIALS AND METHODS

Materials. β-Lg was obtained from Davisco Foods International, Inc. (Eden Prairie, MN). It contained less than 0.1% lactose (dry basis) and less than 0.5% fat (dry basis) and was kept in desiccators containing dry silica gel before use. Fructose, glucose, sorbitol, and glycerol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Alcalase was of food grade from Novozymes A/S (Bagsvaerd, Denmark). TPCK−trypsin was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade.

IMF Model Systems. The IMF model systems contained 45% (w/w) β-Lg, 12.5% (w/w) glucose/fructose/sorbitol, 30% (w/w) glycerol, and 12.5% (w/w) water. Fructose was dissolved in double-distilled water at a weight ratio of 1:1, and then glycerol was added to make a uniform solution. β-Lg was added to the solution, and a dough with a uniform texture was formed. The dough was placed in a plastic cup, covered with the lid, and double-sealed with Parafilm completely around the cup/lid junction to avoid moisture loss. After the samples were equilibrated at room temperature for 1 h, the cups were placed in desiccators with a_W at 0.53 (saturated aqueous magnesium nitrate solution) and then incubated at 25 or 35 °C for up to 49 days. The model systems with glucose or sorbitol were prepared using the same method. The water activity of the model systems was determined with a LAB-H water activity meter (Novasina, Lachen, Switzerland). The water activity of model systems containing fructose and sorbitol was 0.52, and the water activity of the model system containing glucose was 0.54. All of the experiments were duplicated.

Browning Assay. The browning assay[11] was used to determine the progress of the Maillard reaction. The suspension was prepared by adding 250 mg of sample in 10 mL of phosphate buffer (pH 8) under stirring at a speed of 400 rpm for 60 min at room temperature. After adding 12 μL of Alcalase, the solution was placed in a water bath at 55 °C for 15 min. Then, 1 mL of trichloroacetic acid (TCA) (80%, w/v) was added for protein sedimentation and the termination of the enzyme reaction. The solution was filtered, and then its absorbance was measured at 420 nm. All of the experiments were duplicated.

Changes in Proteins by Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was used to detect the change in the molecular weight of protein fractions. A total of 300 mg of sample was dissolved in 10 mL of double-distilled water. The suspension was stirred at a speed of 400 rpm for 60 min at room temperature, followed by centrifugation at 3200g for 30 min. The supernatant was then collected for SDS−PAGE analysis. SDS−PAGE was carried out in 12% separating gel and 4% stacking gel using a Mini-PROTEIN unit (Bio-Rad Laboratories, Hercules, CA). The above solution was diluted 1:1 (v/v) with Laemmli sample buffer (Bio-Rad Laboratories) for non-reducing conditions, while a 950 μL Laemmli sample buffer was mixed with 50 μL of β-mercaptoethanol for reducing conditions prior to use. The mixture was boiled for 3 min and then cooled in an ice bath. A portion of 10 μL of sample was loaded onto the gel. The gels were stained with Coomassie Blue R-250 (Sinopharm Chemical Reagent Co., Ltd.) and destained in a 7.5% acetic acid and 5% methanol solution.

Extent of Glycation. The UPLC−ESI−MS (Platform ZMD 4000 system, Waters Co., Milford, MA), equipped with an Acquity UPLC BEH C4 column (Ethylene Bridged Hybrid, 2.1 × 100 mm, 1.7 μm, Waters Co.), was used to analyze the extent of glycation. A 30 mg sample was added to 10 mL of double-distilled water. The suspension was stirred at a speed of 400 rpm for 60 min at room temperature and centrifuged at 3200g for 30 min. A portion of supernatant was filtered through a 0.45 μm filter membrane. The samples were kept at −20 °C before use, and thawing was conducted at 4 °C. An aliquot of 1 μL was applied to a Waters Acquity UPLC system at a column temperature of 45 °C. Solvent A was aqueous solution containing 0.1% formic acid, and solvent B was 100% acetonitrile. The elution was performed at a flow rate of 300 μL/min using a gradient from 20 to 50% B within 15 min, to 100% B in the following 5 min, back to 20% B within 1 min, and preserving for another 2 min. MS experiments were performed in a positive ionization mode, with the cone voltage at 40 V and the collision energy at 6 eV. The scan m/z range started at 600 and ended at 3000 with a scan time of 1 s and with every 0.02 s interval over the whole course of 23 min. The obtained data were analyzed with the assistance of software MassLynx V4.1 and MassEnt (Waters Co.).

To further compare the glycation extent of two reducing sugars, the average degree of substitution per protein molecule β-Lg (DSP) was calculated according to the following formulation:[32]

$$\text{average DSP} = \left(\sum_{i=0}^{n} i \times I_{(\beta\text{-Lg X}+i\times\text{reducing sugar})}\right) / \left(\sum_{i=0}^{n} I_{(\beta\text{-Lg X}+i\times\text{reducing sugar})}\right)$$

where I is the peak intensity of one variant of β-Lg for various glycated forms, i is the number of attached reducing sugars, and X is variant A or B.

Determination of Glycated Sites. Glycated sites were determined by UPLC−MS^E performed on a Waters SYNAPT MS system (Waters Co.). A total of 50 mg of sample was added to 22.5

mL of double-distilled water. The suspension was stirred at a speed of 400 rpm for 60 min at room temperature. To remove the free reducing sugar in the solution, each portion of 12 mL was dialyzed (molecular mass cutoff of 7 kDa, Union Carbide Co., Danbury, CT) against double-distilled water at 4 °C. After the pH of the sample was adjusted to 8.0 with 0.5 M ammonia, 0.16 mL of 1 M TPCK−trypsin was added to hydrolyze the glycated proteins at 37 °C for 20 h. The enzyme was then inactivated by lowering the pH to 2.0 with 2 M hydrochloric acid. The sample was filtered through a 0.45 μm filter membrane. The samples were kept at −20 °C before use, and thawing was conducted at 4 °C. A Waters Acquity UPLC system (Waters Co.), equipped with an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters Co.), was used for separating the peptides. An aliquot of 10 μL of sample was applied to the Waters Acquity UPLC system at a temperature of 45 °C. The flow rate was kept at 300 μL. Solvent A was 100% formic acid, and solvent B was 100% acetonitrile. The elution was performed at a flow rate of 300 μL/min using a gradient from 0 to 40% B within 55 min, to 100% B in the next 5 min, maintaining at 100% B for another 5 min, then back to 0% B within 1 min, and preserving for another 4 min.

The measurement was performed in a positive ionization mode. The low collision energy was set at 6 V, and the high collision energy was set at 25 V. The capillary and sampling cone voltages were 3.5 kV and 30 V, respectively. The source and desolvation temperature were 100 and 400 °C, respectively. The cone gas flow and the desolvation gas flow were 50 and 500 L/h, respectively. The scan m/z range started at 100 and ended at 2000 with a scan time of 1 s with a 0.02 s interval. The results obtained were analyzed with the assistance of software MassLynx V4.1 and MassEnt (Waters Co.).

A β-Lg three-dimensional (3D) model was established by the software of Swiss-PdbViewer (http://spdbv.vital-it.ch/) to study the glycated sites of β-Lg. The protein structure data originated from PDB file 2Q2M.[33]

RESULTS AND DISCUSSION

Progess of the Maillard Rection by the Browning Assay. The progress of the Maillard reaction was first determined by the browning assay.[11] As shown in Figure 1A, the model system with non-reducing sorbitol was characterized by nearly zero absorbance at 25 °C within the storage time, indicating that the Maillard reaction was negligible. However, the absorbance increased in the systems containing fructose and glucose during storage, revealing the progress of the Maillard reaction between the reducing sugars and β-Lg. The absorbance rose more rapidly for the systems containing glucose than those containing fructose, reaching 0.102 and 0.026, respectively, after the storage of 49 days at 25 °C. The results indicated that glucose had a higher reaction rate than fructose with β-Lg, consistent with the previously reported results,[7,17,34] owing to the higher reactivity of α-hydroxy carbonyl.[17] In the case of storage at 35 °C (Figure 1B), the Maillard reaction appeared more dramatic than that at 25 °C, and the absorbance reached 0.289 and 0.875 for fructose and glucose after 49 days of storage, respectively. This could be explained by the fact that a high temperature contributed to a high Maillard reaction rate, and the reaction sensitivity for a 10 °C increase ranged from four to eight.[35]

Changes in the Protein Fraction Distribution by Gel Electrophoresis. During the experimental period, no significant decrease in β-Lg solubility was observed for the samples stored at 25 and 35 °C. SDS−PAGE was applied under non-reducing and reducing conditions to observe changes in the protein fraction distribution. The sample prior to storage (Figure 2) showed not only a main band of monomeric β-Lg but also a few faint bands of α-lactalbumin, caseins, and BSA, which were the impurities of proteins. No significant change

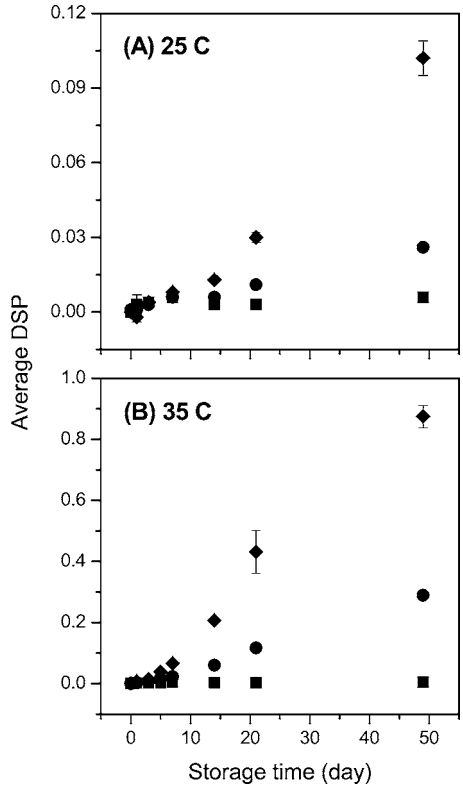

Figure 1. Progress of the Maillard reaction in model systems stored at (A) 25 °C and (B) 35 °C as measured by the browning assay: (■) sorbitol, (●) fructose, and (◆) glucose.

was observed for the model systems containing sorbitol during storage at 25 °C (Figure 2A), but the bands of monomeric β-Lg in the systems with fructose or glucose (panels B and C of Figure 2) migrated upward slowly and appeared broader with the increasing storage time compared to the sample prior to storage. The decreased mobility indicated the increase in the β-Lg molecular weight and, accordingly, the occurrence of glycation. The increased bandwidth indicated the formation of various protein glycoforms.[19] Similarly, the phenomena of migration decreasing and band broadening were also shown in the bands of α-lactalbumin and caseins. The behavior of proteins in the sorbitol system at 35 °C (Figure 2D) was the same as that at 25 °C. The bands of monomeric β-Lg in the systems with fructose or glucose stored at 35 °C (panels E and F of Figure 2) migrated upward slowly and became less intense as the storage time increased. The bands of α-lactalbumin also shifted toward the direction of a high molecular weight and became less intense with the increase of the storage time. In addition, aggregates were observed on the top of the gels at the end of storage. These phenomena suggested that the monomeric β-Lg as well as α-lactalbumin at small quantities were engaged to form dimers or higher molecular weight aggregates involving one or several species of proteins.[5]

In addition, SDS−PAGE with β-mercaptoethanol, a reducing reagent, was further performed (panels G−I of Figure 2). Most aggregates still existed after the addition of β-mercaptoethanol, illustrating that the Maillard reaction induced chemical bonds rather than the disulfide bonds being responsible for the aggregates. When the amount of Maillard-induced aggregates formed by fructose and glucose is compared, glucose was more effective in the formation of aggregates than fructose. Even though it is accepted that ketose produces more aggregates

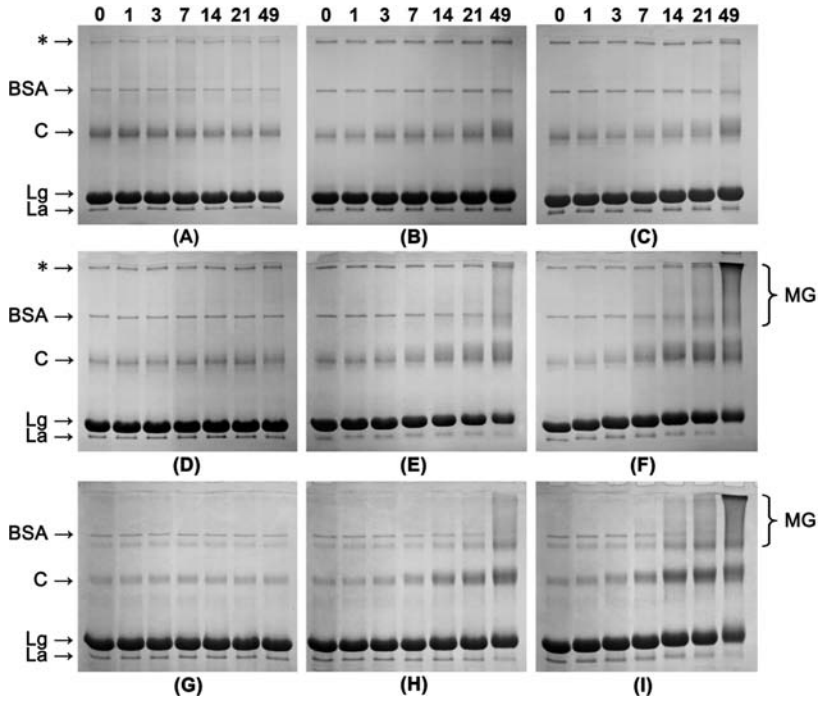

Figure 2. Gel electrophoresis of soluble protein fractions in model systems stored at 25 and 35 °C: (A, B, and C) SDS−PAGE without a reducing reagent for model systems containing sorbitol, fructose, and glucose at 25 °C, respectively, (D, E, and F) SDS−PAGE without a reducing reagent for model systems containing sorbitol, fructose, and glucose at 35 °C, respectively, (G, H, and I) SDS−PAGE with a reducing reagent for model systems containing sorbitol, fructose, and glucose at 35 °C, respectively. The numbers on the top of gels standed for storage days. Protein bands: BSA, bovine serum albumin; C, caseins; Lg, β-lactoglobulin; La, α-lactalbumin; and MG, Maillard-induced aggregation.

through covalent cross-linking than aldose, researchers also reported that glucose would produce more aggregates than fructose at high temperatures, in the presence of pyridoxamine,[6] or in the absence of oxygen.[17] That might indicate the formation of Maillard-induced aggregates being environmentally dependent.

However, there were bands that disappeared after the addition of β-mercaptoethanol. Polymers were formed in the stacking gel in the sample containing glucose stored at 35 °C for 49 days (Figure 2F). Once reducing agents were added, these polymers disappeared (Figure 2I), suggesting they were formed by disulfide bonds. The bands labeled with an asterisk only existed in gels under non-reducing conditions, which might contain immunoglobulin or protein aggregates formed through disulfide bonds in the production of β-Lg. After the addition of the reducing reagents, the protein aggregates formed by disulfide bonding were broken down and the immunoglobulin in the sample was reduced into immunoglobulin heavy chain (IgH) (new bands below BSA) and immunoglobulin light chain (IgL) (the same place as the bands of caseins). However, a further study was necessary before any final conclusion could be made regarding the band with an asterisk.

Extent of Glycation. β-Lg has two variants: A (18 363 Da) and B (18 277 Da). Each variant contains at least 19 potential glycated sites: 15 lysyl residues, 1 N-terminal amino acid (leucyl residue), and 3 arginyl residues. Among the 19 potential glycated sites, the 15 lysyl residues and 1 N-terminal amino acid are the most reactive.

The extent of protein glycation was analyzed by LC−MS. Figure 3 shows the mass spectra of sorbitol systems at 25 °C prior to storage. Two peaks numbered 18 278 and 18 364 corresponded to β-Lg variants B and A, respectively. Besides

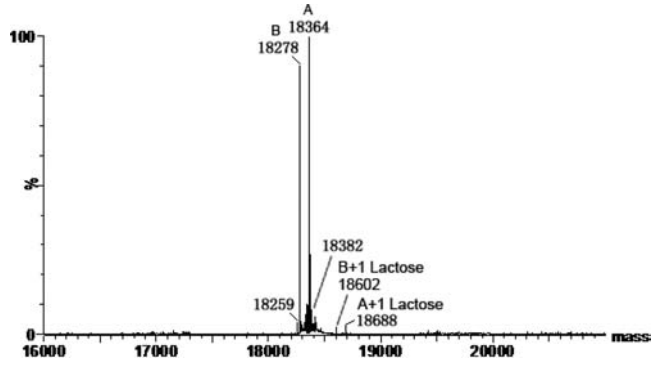

Figure 3. Mass spectrum of the model system containing sorbitol stored for 0 day.

the two major peaks, peaks that numbered 18 259 and 18 382 were also observed, corresponding to a 19 Da mass decrease of variant B and a 18 Da mass increase of variant A, respectively, and these peaks might relate to the products formed through the reaction, such as oxidation and condensation.[25,36] Moreover, there were also two peaks with low signal numbered 18 602 and 18 688, which were the 324 Da mass increases of the two β-Lg variants, probably because of the glycation between β-Lg and lactose presented in the commercial protein powder. The mass spectra of the system containing sorbitol did not change during storage (data not shown), suggesting that no further glycation occurred in the sorbitol system.

The mass spectrum for the fructose system at 25 °C prior to storage (Figure 4A) was similar to that for the sorbitol system. After storage for 3 days, new peaks numbered 18 440 and 18 527 appeared. The 162 Da mass increase suggested that 1 molecule of fructose glycated to variants A and B. The intensity

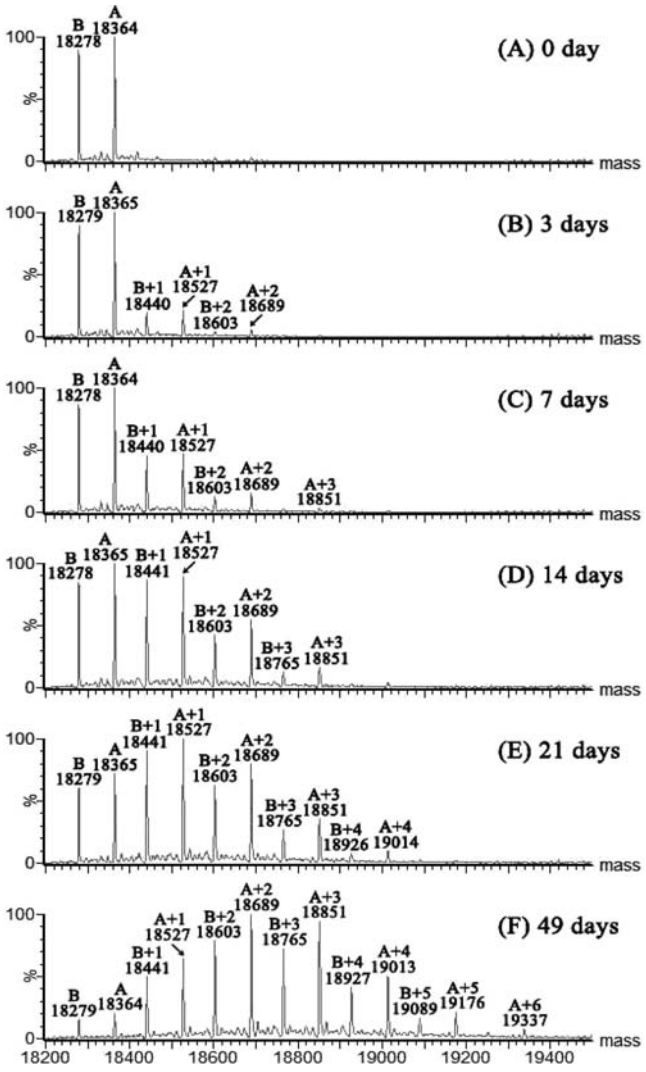

Figure 4. Mass spectra of model systems containing fructose stored at 25 °C.

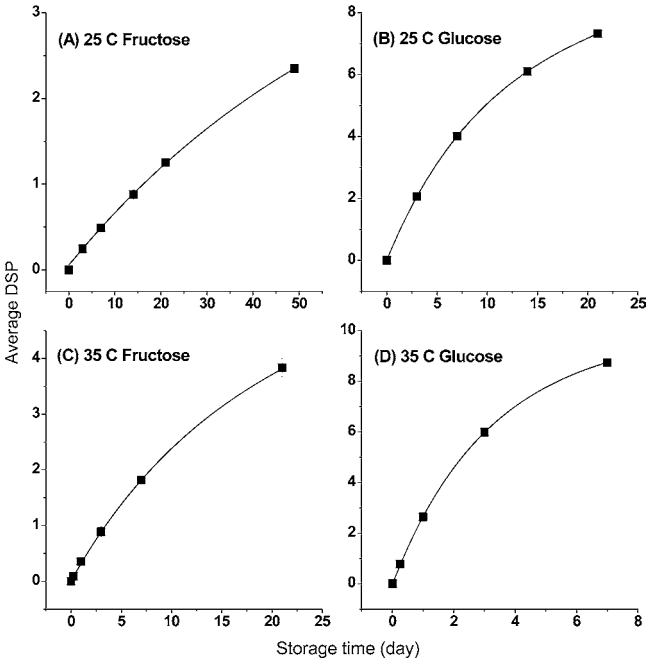

Figure 5. Average DSP curve from glycation.

of peaks numbered 18 603 and 18 689 also increased in comparison to peaks in the mass spectrum of samples prior to storage, suggesting that 2 fructose adducts formed for variants B and A. On the 7th day of storage, a new low-signal peak numbered 18 851 was detected, suggesting that the fructose adducts for variant A increased up to 3, while the fructose adducts for variant B remained 2. The number of fructose adducts increased along with the increasing storage time. In the following storage days, up to 3, 4, and 5 adducts were observed at the 14th, 21st, and 49th days for variant A, and the profile of variant B was the same as that of variant A. The mass spectra for the fructose system at 35 °C and for the glucose system at 25 and 35 °C were shown in the Supporting Information.

To further illustrate the tendency of glycation of β-Lg during storage, the average DSP of variants A and B was calculated separately and found that the two variants had a similar behavior regarding the glycation extent with glucose or fructose. For example, when fructose systems were stored for 3 days, the average DSP for variant A was 0.22, while that for variant B was 0.27. Then, the average of the two variants was used to draw average DSP curves of fructose and glucose systems at 25 and 35 °C (Figure 5). For the glucose systems, more reducing sugars were attached to β-Lg compared to the fructose systems. When incubating at 25 °C, around 2 glucoses were attached per β-Lg at the 3rd day, whereas it cost fructose 49 days to reach a similar average DSP. At the 21st day, the average DSP reached 7.32 and an obvious decrease in the reaction rate occurred. The trend was clearer when the glucose systems were stored at a higher temperature of 35 °C. For the fructose model systems stored at 35 °C for 49 days and the glucose model systems stored at 25 °C for 49 days and at 35 °C for 21 and 49 days, the mass spectrum became too complicated to clearly recognize the peaks representing the glycated β-Lg, suggesting the occurrence of successive reactions. The results of LC−MS confirmed that glucose has a higher reaction activity than fructose, which is consistent with previous studies.[4,6,7,9] In addition, former studies on aldose and ketose also concluded that aldose has a higher glycation reactivity than ketose.[5,8] Even though the two reducing sugars showed a significant difference in the reaction activity, the reduction of the reaction rate of both sugars was observed after more reducing sugars attached to β-Lg. It was probably caused by the reduction of accessibility of potential glycated sites with the progress of protein glycation.

Determination of Glycated Sites. The preference of glucose and fructose for glycation sites was identified. For glucose systems, samples stored at 25 °C for 3 and 21 days were determined, while for fructose systems, samples stored at 25 °C for 3, 7, and 49 days were measured. The freshly prepared model systems were used as a control.

To determine the glycated sites, TPCK−trypsin was employed, which could theoretically recognize the carbon sides of lysyl and arginyl residues but would be inhibited when these two amino acids had proline C-terminal connections. Each variant of β-Lg contains 15 lysyl residues and 3 arginyl residues, but the C-terminal of K 47 was connected with a prolyl residue. Therefore, each variant contains 17 sites of hydrolysis. It was worth noticing that lysyl and arginyl were all potential glycated sites, and it has been reported that, once a reducing sugar was linked, trypsin could not attack the site any more.[37]

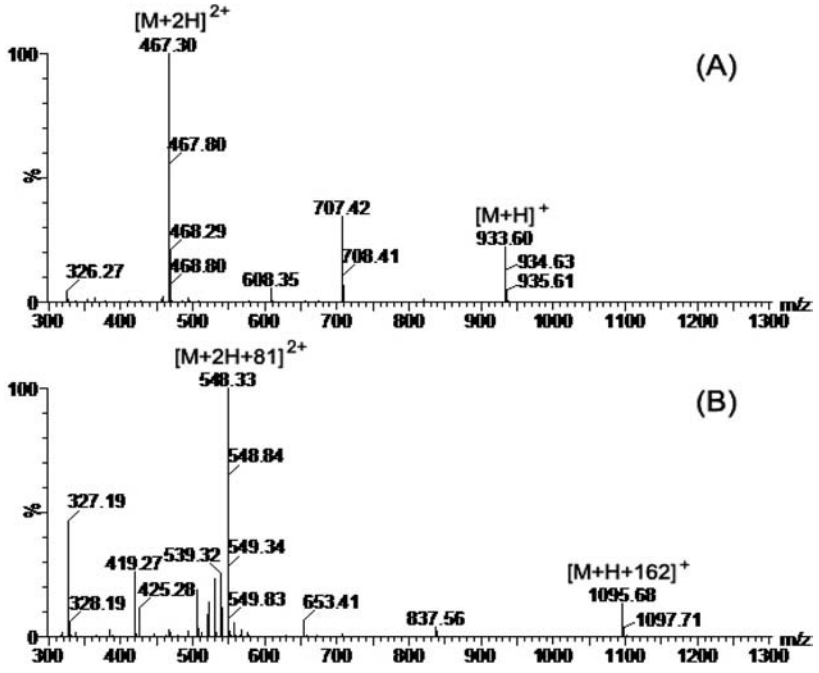

Figure 6. MS spectra for peptide 1−8 of β-Lg: (A) glucose system stored for 0 day and (B) glucose system stored at 25 °C for 7 days.

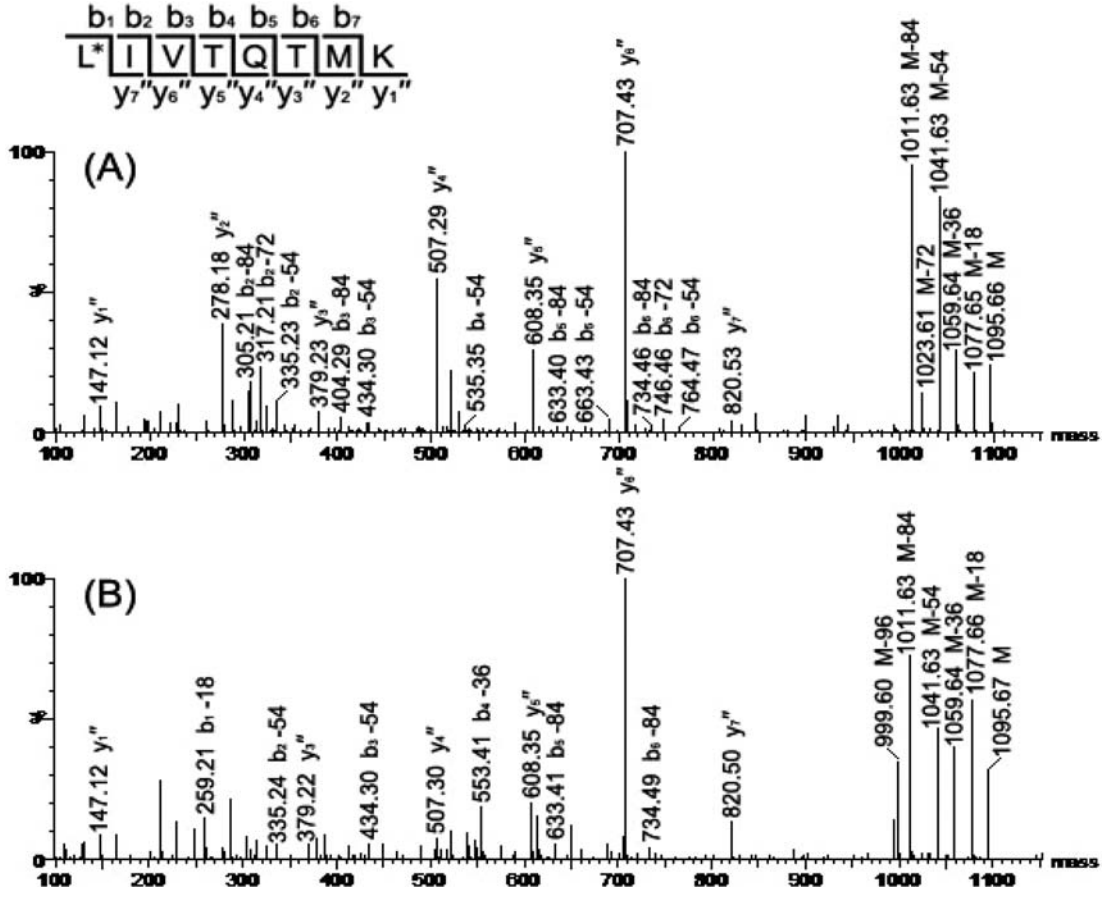

Figure 7. Deconvoluted MS^E spectra for the glycated peptide 1−8 stored at 25 °C for 7 days: (A) for glucose and (B) for fructose. Leu 1 is identified as the glycated site.

The glycated sites were determined by LC−MS^E. MS data were first provided to identify the probably glycated peptides. Theoretically, if a peptide was glycated, the corresponding m/z peaks with 1, 2, or 3 ions would display an increase of 162, 81, or 54, respectively. For example, the m/z peaks of native peptide 1−8 (Figure 6A) were at 467.30 for $(M + 2H)^{2+}$ and 933.60 for $(M + H)^+$, while the m/z peaks of peptide 1−8 stored for 7 days at 25 °C (Figure 6B) were at 548.33 for (M +

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2H + 81)$^{2+}$ and 1095.68 for (M + H + 162)$^{+}$, indicating that peptide 1−8 was glycated by 1 molecule of glucose. Further studies on fragment ions of glycated peptides in the MS^{E} spectra were applied to identify the accurate glycated sites. A previous study[38] showed that the attached sugars had the tendency to lose neutral fragments to form corresponding b and y′′ fragment ions. Well-controlled collision energy would release these neutral losses to determine the glycated amino acid. For example, in the MS^{E} spectra of glucosylated peptide 1−8 (Figure 7A), 18, 36, 54, 72, and 84 Da neutral losses were found. The former four mass losses indicated the consecutive water losses varying from 1 to 4, and the later mass loss was led by the separation of 3 water molecules and an additional formaldehyde molecule.[29,39] A full series of y′′ ions was detected, which suggested that no glycation happened to the amino acid sited 2−8. The results were further confirmed by the b fragment ions. For the glycated peptide 1−8 reacted with fructose (Figure 7B), a unique neutral loss of 96 Da[29] was detected, which distinguished fructose from glucose. In this way, a total of 9 glycated sites were identified (Table 1).

Table 1. Glycated Amino Acid Residues of β-Lg in Model Systems Containing Fructose or Glucose

	glycated amino acid residues								
samples[a]	L 1	K 47	K 70	K 75	K 77	K 83	K 91	K 100	K 135
F-3d	+						+		
F-7d	+	+					+		
F-49d	+	+	+		+	+	+	+	
G-3d	+	+	+	+	+	+	+	+	
G-21d	+	+	+	+	+	+	+	+	+

[a]F and G represent fructose and glucose.

For the fructose systems stored at 25 °C for 3 and 7 days, 2 and 3 glycated sites were detected, which fitted well with the results of the glycation extent. For 49 days, 7 glycated sites were identified, even though there are a maximum of 6 attachments detected in the determination of the glycation extent. It may be explained by the low amount of glycated β-Lg with 7 attachments compared to the amount of that with 2 attachments. This was the same as the glucose systems stored at 25 °C for 3 days (see the Supporting Information), which owned a similar LC−MS spectrum to the one for fructose systems stored at 25 °C for 49 days. For the glucose systems stored at 25 °C for 21 days (see the Supporting Information), the result of the glycation extent showed that the attachment number varied from 4 to 11 and the most abundant product was glycated β-Lg with 7 attachments for both variants A and B. However, only 9 glycated sites were identified. Besides the 9 glycated sites, other potential glycation sites, such as K 14, K 60, K 69, K 101, K 138, and K 141[12,20,25,27,30,37,39−41] and R 40, R 124, and R 148,[20] were not detected in this study. It was possible because of the low reaction activity or the limited availability of these amino acid residues. In addition, there is also the possibility that some amino acid residues were indeed glycated but not detected. The ineffective detection can be explained by four main reasons. First, the amounts of peptides containing glycated residues were not enough to be detected.[15] Second, the peptides resisted the collision energy, so that even though some suspected glycated peptides were detected in the MS, no further fragmentation information was available. Third, the ionization properties of these peptides decreased after glycation.[25] Fourth, the existence of disulfide bonds made detections less effective.

As for the 9 potential glycated sites, the reaction activities varied. When all of the measured data in the present study were compared (Table 1), the ranking order from high to low for the 9 detected glycated sites was L 1, K 91 > K 47 > K 70, K 77, K 83, K 100 > K 75 > K 135. The data also suggested that glucose and fructose have a similar preference on the glycation sites of β-Lg. A 3D model of β-Lg (Figure 8) was built in an attempt to

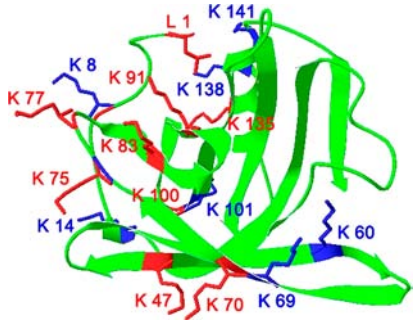

Figure 8. Three-dimensional model of monomer β-Lg. Green, framework of β-Lg; red, glycated amino acid residues detected in the present study; and blue, unglycated lysine residues in the present study.

give an explanation from the angel of the spatial structure. Viewed from the 3D model, several reasons were responsible for the reactivity of the potential glycated sites. First of all, the position of the amino acid residues was important. For example, L1 was located in the N-terminal, which resulted in its highest reactivity. Besides, the exposure of amino acid residues was another reason for the differences among the potential sites. Although K 70, K 60, and K 69 were all located on the β-strand D, the side chain of K 70 was exposed outside, making it accessible to the reducing sugars, whereas the side chains of K 60 and K 69 pointed into the cavity, leaving them less likely to be glycated.[42] The low reactivity of K 14 can also be explained by the fact that it was buried in the tertiary structure of β-Lg.[42] In addition, the nearby charged amino acid affected the formation of the Amadori compound.[43] For example, the solvent exposition of K 47 was lower than K 8, but K 47 was more active than K 8.[20,44] Fogliano et al.[43] explained this by comparing the proximity of the basic residue with a positive charge: K 47 was very close to K 70, while K 8 was far away from any other positive-charged amino acid. The activities of other amino acids, such as K 77 and K 100, approaching another lysyl residue could also been explained in this way.

In conclusion, glucose was more reactive in the Maillard reaction activity than fructose in IMF systems, leading to more nutrition loss and browning color. Although these two sugars have significant differences in the reaction activity, their preferences on the glycation sites are similar. The ranking order from high to low for the 9 detected glycated sites was L 1, K 91 > K 47 > K 70, K 77, K 83, K 100 > K 75 > K 135 for both glucose and fructose. As for β-Lg, variants A and B had a similar behavior regarding the glycation extent with glucose and fructose. In addition, in comparison to the browning assay and SDS−PAGE, LC−MS and LC−MS^{E} were more sensitive and accurate in analyzing the protein glycation, the early stage of the Maillard reaction.

■ ASSOCIATED CONTENT

Supporting Information

Mass spectra of model systems containing fructose stored at 35 °C (Figure S1), glucose stored at 25 °C (Figure S2), and glucose stored at 35 °C (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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