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Evaluation of the Site Specific Protein Glycation and Antioxidant Capacity of Rare Sugar−Protein/Peptide Conjugates

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Protein−sugar conjugates generated in nonenzymatic glycation of α-lactalbumin (LA) with rare sugars [D-allose (All) and D-psicose (Psi)] and alimentary sugars as controls [D-glucose (Glc) and D-fructose (Fru)] were qualitatively determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Mass spectra revealed that the extent of glycation at lysine residues on LA with D-aldose molecules was very much higher than that of glycation with D-ketose molecules. To identify the specific site of glycation, the peptide mapping was established from protease V8 digestion, using a combination of computational cutting of proteins and MALDI-TOF-MS. As compared to peptide mapping, three and seven glycation sites were located in the primary structure of LA−ketose and LA−aldose conjugates, respectively. On the other hand, the antioxidant activities of protein−sugar conjugates and their peptic hydrolysates were investigated by 1,1-diphenyl-2-picrylhydrazyl radical scavenging method. The antioxidant activities of proteins/peptides glycated with rare sugars were significantly higher than those modified with the control sugars. The results indicated that the glycation degree and position were not markedly different between rare sugar and corresponding control sugar, but the antioxidant properties of protein and its hydrolysate were significantly enhanced by modifying with rare sugar.

KEYWORDS: α-Lactalbumin; rare sugar; glycation; antioxidant activity; peptide-mapping; MALDI-TOF-MS

INTRODUCTION

The nonenzymatic reaction of a reducing sugar and the amino groups of a protein or a peptide is called the Maillard reaction (MR) or glycation. The reaction proceeds through two stages. In the early stage, a sugar reacts with the amino groups of protein/peptide to form a stable Amadori and Heyn's product, via a labile Schiff base. In the advanced stage, many different, complex reactions occur, and consequently, brown, cross-linked fluorescent products are produced (*1*). Protein glycation reactions, the subsequent modification of protein functionality, and the physiological and pathological consequences of protein glycation have been the subject of much investigation.

The MR, which is one of the most important phenomena occurring in food during processing and storage, represents an interesting research area. It was indicated that the glycated proteins through the MR could be added as functional ingredients to foods to improve emulsion and gelation and alter flavor, appearance, and texture (*2*−*4*). It had been reported that the modified egg white protein with a rare hexose, D-psicose (Psi), could develop more excellent heat-induced gelling properties, such as gel strength, viscoelastic property, and water-holding capacity (*5*, *6*). The role of glycated proteins in the diet as physiologically active components has also been increasingly acknowledged. Many studies have reported some beneficial effects associated with the Maillard reaction products (MRPs), including the formation of compounds with antioxidant (*6*, *7*), anticarcinogenic, and antimutagenic properties (*9*, *10*). Chevalier et al. (*11*) reported that proteins modified with ribose and arabinose showed a stronger radical scavenging activity and suggested that the glycated protein could be used in formulated food as functional ingredients with a radical scavenging activity able to delay deterioration due to oxidation.

Rare sugars are defined as "monosaccharides and their derivatives that are rare in nature". Because rare sugars are not abundant in nature and are difficult to prepare by chemical methods, their biological activities still are not very well-known. In recent years, Bhuiyan et al. reported a new enzymatic method of D-allose (All) production from Psi that acted as a raw material using L-rhamnose isomerase (*12*). Furthermore, Takeshita et al. reported an improved method for the large-scale production of Psi from D-fructose (Fru) using D-tagatose 3-epimerase (*13*). These findings provide the possibility for a rare sugar study and utilization of their biological activities. As reported in the recent literature, a great deal of studies were concentrated on the role of rare sugars for a variety of uses, such as potential inhibitors of various glycosidases (*14*), low-calorie carbohydrate sweeteners, and bulking agents (*15*, *16*), and improving the

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clinical effect as an immunosuppressant on allogenic orthotopic liver transplantation in rats (*17*). It has been suggested that Psi might be useful as a noncalorie sweetener in the food industry for obese people as an aid for weight reduction (*18*). In our previous study, it was reported that protein−All/Psi complexes exhibited a stronger antioxidant activity and a greater aggregation and browning/fluorescence development than protein−D-glucose (Glc)/Fru complexes, suggesting that protein modified with rare sugar as a functional ingredient could be applied in food (*6*, *19*). However, up to now, no attention has been paid to the primary structural modifications of protein with rare sugars by nonenzymatic glycation, as well as the effect of this modification on functional properties of glycated proteins.

In the present investigation, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to determine α-lactalbumin (LA) modifications formed by MR with two rare sugars and two control sugars at different incubation times. To solve the modifications of structural level, the peptide mapping was established from a V8 proteolysis experiment combined with computational cutting of proteins and MALDI-TOF-MS analysis. Moreover, the glycated peptides derived from the digestion were used to characterize and the glycation sites could be used to locate the primary structure of LA−sugar conjugates. To understand the effect of primary structural modifications on functional properties, the antioxidant activity of sugar−protein/peptide conjugates was investigated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method.

MATERIALS AND METHODS

Materials. Psi and All were obtained from Kagawa Rare Sugar Research Center (Kagawa, Japan). V8 protease (Staphylococcal Serine Proteinase, EC 3.4.21.19), Glc, and Fru were purchased from Wako Pure Chemical Industries (Osaka, Japan). LA (type 1: from bovine milk) was obtained from Sigma-Aldrich. Trypsin (TPCK-modified, T 1426) and pepsin (porcine stomach mucosa, pepsin A, EC 3.4.23.1) were obtained from Sigma Chemical Co. All other chemicals used were guaranteed reagent grade.

Glycation of LA. Glycation of LA was carried out with two rare sugars and two alimentary sugars. LA was dissolved in 10 mM carbonate buffer (pH 9.0) at a protein concentration of 5% (w/v) with the sugars (16.4% of the protein dry weight); that is, a molar ratio is about 1:13 of protein vs sugar. The solutions were lyophilized. The mixed samples were incubated at dry state at 50 °C and 55% relative humidity (RH) for up to 48 h (an interval of 12 h) in an incubator (LHL-113, Espect Co., Japan). For these procedures, control experiments (Ct) were carried out with no added sugars, and three separate experiments were conducted. The protein content of samples in the present study was determined by the Lowry method (*20*).

Proteolytic Digestion of Intact and Glycated LA. The intact, control, and glycated samples (1 mg/mL) were first reduced by incubation with 5.0 mM dithiothreitol (DTT) at 30 °C for 2 h in 20 mM phosphate buffer (PB, pH 7.8), containing 1.0 mM ethylenediaminetetraacetic acid. The reduced sample was directly proteolyzed with V8 protease at 37 °C for 16 h. A portion of reduced samples was incubated with 15.0 mM iodoacetamide (IAM) at 30 °C for 2 h in the dark to block the free sulfhydryl (SH) groups from the reduced disulfide (SS) bonds. Excess IAM and DTT were removed by dialysis in 20 mM PB overnight. The modified samples with IAM were further digested with V8 protease under the same conditions as described above. In addition, the sample treated in the same pattern was dissolved in 50 mM ammonium hydrogencarbonate (pH 7.8) and digested with trypsin at 37 °C for 3 h. The enzyme/substrate (E/S) molar ratio was 1/100 and 1/50 for V8 protease and trypsin, respectively.

Control and glycated LA (10 mg/mL) were dissolved in 0.25 N acetic acid buffer (pH 2.6) for peptic hydrolysis. Porcine pepsin, previously solubilized in distilled water (1 mg/mL), was added to the sample solution at an E/S molar ratio of 1/100. The reaction mixtures were incubated at 37 °C for 2 h and then lyophilized. The extent of hydrolysis was monitored by MALDI-TOF-MS and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

MALDI-TOF-Mass Spectrometry. MALDI-TOF MS analysis was performed on an Autoflex (Bruker Daltonics, Germany) mass spectrometer operated in a linear and reflector, positive ion mode with an N_2 laser (337 nm). The samples were diluted with 0.1% TFA (trifluoroacetic acid) aqueous solution at a last concentration of about 10 pmol for protein and 5 pmol for peptide. 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid, dissolved in TA solution (0.1% TFA with 33% acetonitrile) at saturation state, were used as the matrices for protein and peptide analysis, respectively. One microliter of sample solution was added to 4 μL of the matrix solution, and 1 μL of this mixture was spotted onto a stainless steel target, air-dried, and subjected to mass determination. Spectra were averaged from about 300 laser shots to improve signal-to-noise levels. External mass calibration was performed by use of a standard protein/peptide mixture (Bruker, Daltonics).

Gel Electrophoresis. Maillard-reacted LA samples and their hydrolysates were analyzed on 15% SDS−PAGE in the presence of 2-mercaptethanol followed by staining with Coomassie Brilliant Blue, according to the Laemmli method (*21*).

Determination of Available Amino Groups. The quantity of available amino groups was determined according to the OPA (*o*-phthalaldehyde) method (*22*). To a 50 μL aliquot of samples containing 2 mg/mL LA was added 1 mL of OPA reagent. The absorbance was read at 340 nm after a minimal delay of 2 min at room temperature. A calibration curve was obtained by using 0.25−2.00 mM glycine as a standard.

Scavenging Effect on DPPH Radical. The scavenging effect of the protein and its hydrolysate on the DPPH radical was estimated according to Chevalier method (*11*) with some modifications. The sample solutions of proteins and peptides were dissolved in distilled water, and 0.1 mL of each sample was added to 0.9 mL of DPPH solution (Sigma, with a concentration of 0.1 mM) in methanol solution. The mixture was shaken vigorously and allowed to stand for 30 min in the dark. Then, the absorbance of the resulting solution was measured at 517 nm using UV mini-1240 spectrophotometer (Shimadzu, Co., Japan). The DPPH scavenging effect (%) was calculated as:

$$[(OD_{517control} - OD_{517sample})/(OD_{517control})] \times 100\%$$

For all experiments, distilled water instead of sample solution was used as a control. Trolox (a water soluble vitamin E analogue) solution was used as a standard, and triplicates were analyzed for each sample. Mean values are plotted for the table and figures, and standard deviation and *t*-tests are calculated using data of each replicate. A two-tailed *p* value lower than 0.05 is considered to be significant.

RESULTS AND DISCUSSION

Determination of Extent and Rate of Protein Glycation. The LA protein was incubated with four reducing sugars up to 48 h (an interval of 12 h) at 50 °C and 55% RH. To assess the occurrence of covalent LA−sugar conjugates, the extent and rate of protein glycation were analyzed by MALDI-TOF-MS spectra. First, the intact LA was analyzed by MALDI-TOF-MS spectra, and this procedure was repeated three times. A mean mass/charge (*m*/*z*) value of 14179 ± 5 for intact LA was observed (**Figure 1**). Mass value of control sample obtained by incubation of LA without reducing sugar showed no variation as compared to that of intact LA. As the addition of a hexose molecule to the protein leads to a mass increase of 162 Da, it is possible to calculate the number of reducing sugar molecules condensing on LA as compared to the molecular weight of intact LA (*m*/*z* 14179). The samples were drawn after 24 h of incubation and analyzed by MS spectra (**Figure 1A**). In addition to the unmodified protein at *m*/*z* 14179, main peaks at *m*/*z*

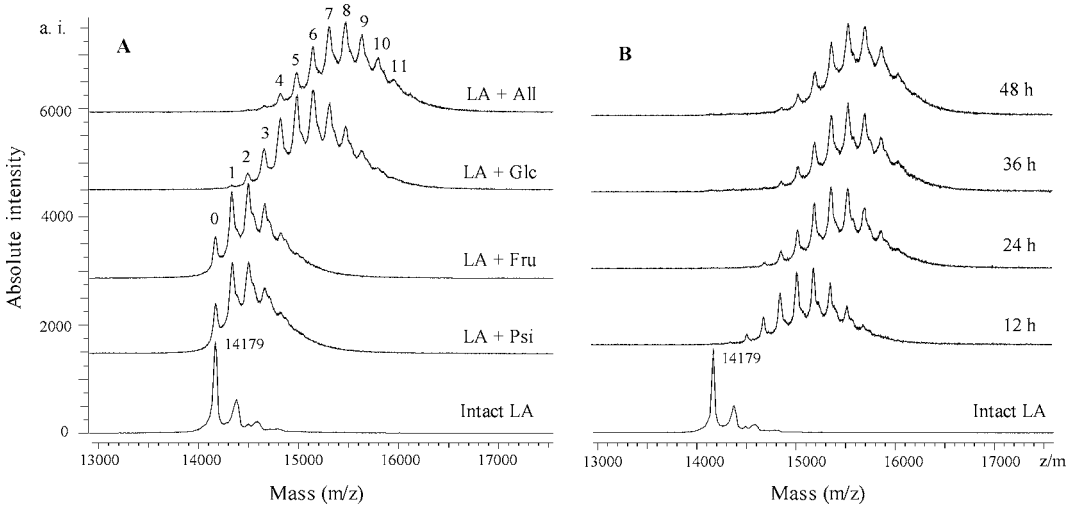

Figure 1. MALDI-TOF-MS spectra of intact and glycated LA. (**A**) LA samples were incubated with Fru, Psi, Glc, and All at 50 °C and 55% RH for 24 h. The peak number means the number of sugar molecules condensing on LA. (**B**) LA samples were incubated with All for 12, 24, 36, and 48 h, respectively.

14338, 14499, 14664, and 14825 were observed in LA−Psi and LA−Fru conjugates, suggesting that 1−4 ketose molecules condensed on LA after 24 h of incubation. MS spectra also showed the mass ranges at *m*/*z* 14495−15792 and 14820−15951 for LA−Glc and LA−All conjugates, respectively. This observation indicated that 2−10 Glc and 4−11 All molecules condensed on protein (**Figure 1A**). These results showed that LA exhibited a different degree of glycation for aldo-hexose and keto-hexose, fitting the large structural differences between the two reducing sugar species. In general, aldoses are considered to be more reactive than ketoses because of their more electrophilic carbonyl group (*23*, *24*).

A time−course study on LA glycation was performed using four reducing sugars for 12, 24, 36, and 48 h of incubation to investigate the rate of glycation. As an example, **Figure 1B** showed the MS spectra patterns of LA−All conjugates in this time course. The rate of glycation was very fast during the period of 12 h of incubation and then slightly increased up to 24 h of incubation. Furthermore, prolonging the incubation time to 48 h did not have a marked effect on the MS spectra profile of glycated monomeric LA, and at that incubation time, the dimeric peak was markedly observed (data not shown). This dimeric pattern was consistent with the SDS−PAGE profile shown in **Figure 6A**. The results suggested that the reaction of LA condensed with four reducing sugars occurred principally during 24 h of incubation. Given the above results, the samples obtained from 12 and 24 h of incubation were employed in subsequent digestion experiments.

MR involves, first, the formation of a Shiff base after the reaction of the reducing sugar with the amino group of a protein. In our preliminary experiment, the results proved that the guanidine group of the Arg_{10} residue containing on LA was not glycated (not shown). Thus, only the α-NH_2 of the N-terminal amino acid and ϵ-NH_2 of Lys residues can be modified, which amounts to 13 residues in LA protein. In this study, the free amino groups of glycated LA were also detected by OPA measurement. The results showed that the numbers of glycations for 24 h of incubation samples were the averages of 2.0 for Psi, 2.2 for Fru, 6.1 for Glc, and 7.4 for All. From these results, the glycation of LA with aldose and ketose shows some peculiar differences in terms of the number of condensed sugar molecules.

Establishment of Peptide Mapping. To obtain information about the site specific modification of the Lys moieties, the peptide mapping was first established from the V8 proteolysis experiment. V8 protease cleaves C-terminal Glu and Asp peptide bonds in phosphate-buffered medium. However, scission of Asp-X is less favored than that of Glu-X, which thus yields the major fragments (*25*). Native LA was digested by V8 protease to obtain defined fragments of lower molecular mass (500−3500 Da). The primary sequence of LA was downloaded from Swiss protein database (http://www.expasy.com/), and the sites of proteolytic cleavage could thus be located in the primary structure. Computational methods were used to predict the peptides generated from the hydrolysis of LA by V8 protease, and predicted sequences of peptide fragments and their theoretical mass values are shown in **Table 1**. By theoretically digesting LA with V8 protease, we were able to link an observed peak to a defined peptide fragment or amino acid sequence. The results indicated that 10 major peptide fragments could be identified from their masses, the known sequences of LA, and cleavage sites of V8 protease (**Table 1**). However, the fragment (at *m*/*z* 721.4, sequence 2−7) could not be detected, and the fragment (Lys_{79}−Glu_{87}) at *m*/*z* 1080.2 was detected slightly. For a variety of reasons, it is normal for only a subset of the potential hydrolysates from any protein to be detected, particularly when the peptides are ionized directly from unseparated mixtures in which there may be competition for the available protons. Furthermore, to probe the N-terminal peptide, the tryptic digestion was performed in the same procedure. The result showed that two fragments associated to N-terminal peptides EQLTK (at *m*/*z* 618.3, sequence 1−5) and CEVFR (at *m*/*z* 653.3, sequence 6−10) were identified, respectively (**Figure 2**).

Some proteins containing SS bonds maintain a rigid higher-order structure and show different sensitivities to digestion. Furthermore, their peptide digestion map is quite different from that reported in the protein database; consequently, identification by peptide mass mapping is not effective (*26*). The bovine LA consists of 123 amino acid residues, containing four SS bonds (Cys_6−Cys_{120}, Cys_{61}−Cys_{77}, Cys_{73}−Cys_{91}, and Cys_{28}−Cys_{111}). In our preliminary experiment, the reduced degree of LA directly affected protein proteolytic digestion by V8 protease and trypsin. To assess the reduced degree and to further verify the peptide mapping, SS bonds of LA were reduced with DTT; subse-

Table 1. MALDI-TOF-MS Analysis of Peptides Obtained from the Protease V8 Digestion of Intact and Glycated LA with Four Reducing Sugars

no.	position	peptide sequence[a]	M_r theoretical[a]	M_r observed	modified peptide with IAM (ΔM)[b]; site of Cys	glycated peptide with All and Glc (ΔM)[c]; site of Lys	glycated peptide with Psi and Fru (ΔM)[c]; site of Lys
1	2–7	QLT**K**CE	721.4	?			
2	8–11	VFRE	550.3	550.1			
3	12–25	L**K**DL**K**GYGGVSLPE	1474.0	1475.8		1638.0 (ΔM, 162.2); 1800.1 (ΔM, 324.3); K_{13}*[d] and K_{16}	1638.0 (ΔM, 162.2); K_{13}*
4	15–25	L**K**GYGGVSLPE	1119.6	1119.7		1281.6 (ΔM, 161.9); K_{16}	
5	26–37	WVCTTFHTSGYD	1416.6	1416.7	1474.5 (ΔM, 57.8); C_{28}		
6	26–49	WVCTTFHTSGYDTQAI VQNNDSTE	2716.0	2716.3	2774.0 (ΔM, 57.7); C_{28}		
7	50–78	YGLFQINN**K**IWC**K**DDQ NPHSSNICNISCD	3370.5	3370.8	3544.4 (ΔM, 173.6); C_{61}, C_{73}, C_{77}		
8	79–87	**K**FLDDDLTD	1080.0	?			
9	89–97	IMCV**KK**ILD	1062.6	1062.5	1120.1 (ΔM, 57.6); C_{91}	1225.0 (ΔM, 162.5); K_{93} or K_{94}	
10	98–113	**K**VGINYWLAH**K**ALCSE	1832.0	1832.2	1890.5 (ΔM, 58.3); C_{111}	1994.2 (ΔM, 162.0); 2156.1 (ΔM, 323.9); K_{98} or K_{108}	1994.1 (Δ, 161.9); K_{98} or K_{108}
11	114–121	**K**LDQWLCE	1033.0	1033.6	1091.9 (ΔM, 58.3); C_{120}	1196.0 (ΔM, 162.4); K_{114}	1196.1 (ΔM, 162.5); K_{114}*
12	114–123	**K**LDQWLCE**K**L	1274.0	1273.6		1436.0 (ΔM, 162.4); 1597.8 (ΔM, 324.2); K_{114} and K_{122}*	

[a] The peptide sequences and their theoretical masses were obtained from Swiss protein database. [b] ΔM means the difference from nonmodified and modified peptides by IAM. [c] ΔM means the difference from nonglycated and glycated peptides with reducing sugars. [d] An asterisk means the data from samples incubated for 24 h and other data from glycated samples for 12 h.

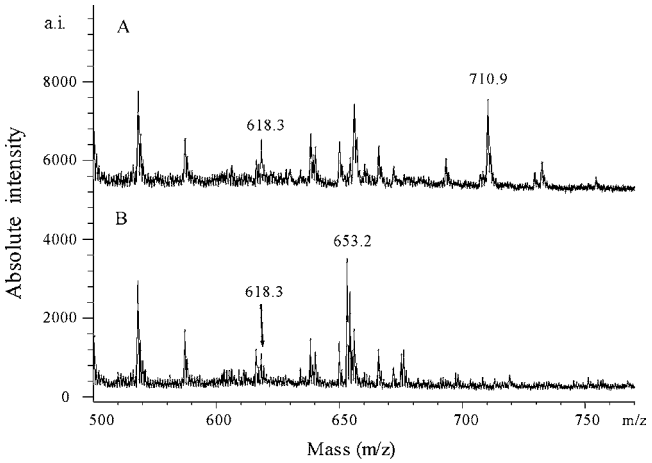

Figure 2. MALDI-TOF-MS spectra of tryptic peptides. The reduced LA was modified with IAM (**A**) and without IAM (**B**). The peaks at *m*/*z* 618.3 and 653.3 represent N-terminal peptides associated to sequences 1–5 and 6–10, respectively. The peak at *m*/*z* 710.9 represents a modified fragment (sequences 6–10) with IAM.

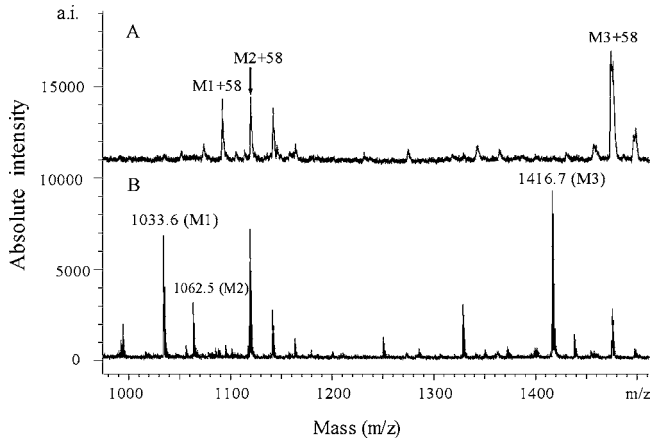

Figure 3. MALDI-TOF-MS spectra of protease V8 digests of intact LA. The reduced LA protein was modified with IAM (**A**) and without IAM (**B**). The positions of fragments corresponding to major peaks were shown in **Table 1**.

quently, the reduced cysteine residues were alkylated with IAM. The cysteine-containing peptides were characterized by the same analytical approaches as described above. **Figure 3** showed MS spectra of V8 protease digests of intact LA modified with and without IAM in the mass range from 1000 to 1500. In the case of LA modified with IAM, the peaks of fragments at *m*/*z* 1416.7 (sequence 26−37), 1062.5 (sequence 89−97), and 1033.6 (sequence 114−121) disappeared, and the new peak at *m*/*z* 1474.5, 1120.1, and 1091.9 with an excess mass of 58 Da appeared (**Figure 3A**). Thus, it was suggested that Cys_{28}, Cys_{91}, and Cys_{120} residues could be modified with IAM. In addition, **Figure 2** showed that Cys_6-containing peptide at *m*/*z* 653.2 was also modified by IAM with a mass difference of 58 Da. Similarly, mass differences in the spectra corresponding to 58 Da (and multiples thereof) revealed that other several peptides have been modified by IAM, and these cysteine-containing peptides identified were summarized in **Table 1**. From these results, it was clearly indicated that four SS bonds had been reduced completely by DTT under this reduced condition.

Characterization of Glycated Peptides and Specific Sites. With the aim to identify the amino acids glycated following MR, the protein−sugar conjugates incubated for 12 and 24 h were subjected to V8 protease digestion and examined by MALDI-TOF-MS. The identity of both native and glycated peptides was unequivocally established by comparing the *m*/*z* values of the hexose moiety (162 Da). The potential glycation sites could thus be located on primary structures without peptide isolation.

MALDI-TOF-MS spectra showed that the absolute intensity of some peaks derived the glycated sample results in a marked

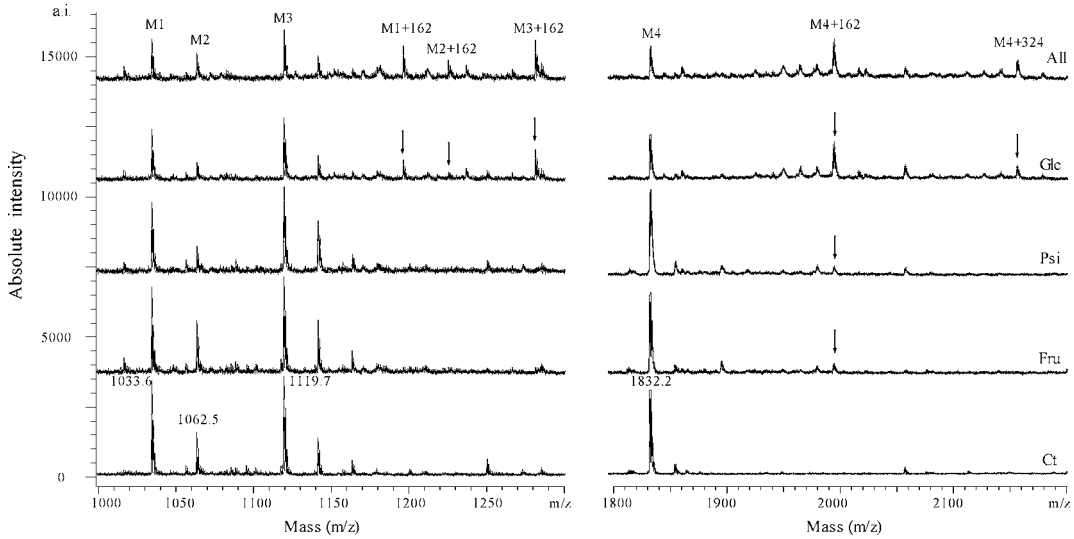

Figure 4. MALDI-TOF-MS spectra of glycated and control (Ct) samples digested with protease V8. The samples were incubated with four reducing sugars (Fru, Psi, Glc, and All) at 50 °C and 55% RH for 12 h. M1, M2, M3, and M4 represent the molecular masses at *m*/*z* 1033.6, 1062.5, 1119.7, and 1832.2, respectively.

decrease, followed by an appearance of some new peaks as compared to the nonglycated sample. It might be inferred that these peptides observed in a decrease of intensity could be partially substituted by the glycated analogues. As an example, the spectra of the region between *m*/*z* 1000 and 1300 were analyzed (**Figure 4**). According as peptide mapping, this region contained three major fragments (corresponding to the sequences 114−121, 89−97, and 15−25) with the molecular masses of *m*/*z* 1033.6, 1062.5, and 1119.7, respectively. In the case of LA−All and LA−Glc conjugates, three new peaks could be distinguished with the molecular masses of *m*/*z* 1196.0, 1225.0, and 1281.6, respectively. These components could be associated to three fragments at *m*/*z* 1033.6, 1062.5, and 1119.7 resulting from the addition of single hexose moiety (ΔM = 162 Da) to peptide amino groups through the MR, respectively. It clearly exemplifies the proteins glycated with aldo-hexose at the sites of Lys_{114} and Lys_{16}, respectively. However, the glycation sites of the fragment at *m*/*z* 1062.5 (sequence 89−97) cannot be located specifically, because this fragment contains two Lys residues (Lys_{93} and Lys_{94}).

Figure 4 showed the equivalent spectra of nonglycated LA and glycated LA with four sugars in a mass range from *m*/*z* 1800 to 2200. In the case of All− and Glc−LA conjugates, the peak at *m*/*z* 1832.2 (sequence 98−113) markedly decreased, and two additional peaks corresponding to masses of *m*/*z* 1994.2 (ΔM = 162.0) and 2156.1 (ΔM = 323.9) appeared, respectively. This peptide containing the two potential glycation sites Lys_{98} and Lys_{108} was clearly identified. In the case of LA−Psi and LA−Fru conjugates, only one new peak appeared at *m*/*z* 1994.1 (ΔM = 161.9), suggesting that the modification with only one ketose molecule seemed very plausible. However, it was also not possible to distinguish a specific Lys position for the ketose adducts in this case. For more information on glycated peptides, the mass spectra obtained with molecular masses ranging from 500 to 3500 Da for glycated and nonglycated samples were compared and the most important results are listed in **Table 1**.

In the next step, trypsin was used for partial enzymatic hydrolysis of glycated LA. Trypsin cleaves C-terminal Lys and Arg bonds. As shown in **Figure 2**, one of the tryptic peptides at *m*/*z* 618.3 corresponding to the N-terminal fragment contains one α-NH_2 of Glu_1 and one ϵ-NH_2 of Lys_5. However, the peaks of peptides corresponding to addition of single or double hexose molecules were not found for all four protein−sugar conjugates in MS spectra of tryptic digests (data not shown), suggesting that glycation did not occur at such two free amino groups of the peptide. On the other hand, as already reported by Smales et al. for fructosylated lysozyme, trypsin does not cleave at the modified positions, suggesting that Lys-modified protein peptides are formed only from tryptic cleavage at Arg-X (*27*). In the present study, MS spectra of tryptic peptides showed clearly two fragments associated to N-terminal peptides at *m*/*z* 618.3 (sequence 1−5) and at *m*/*z* 653.3 (sequence 6−10). It was further proved that ϵ-NH_2 of Lys_5 could not be modified. Thus, it could be inferred that the N-terminal two free amino groups were not the potential glycation sites. The result agreed well those reported by Tagami et al. who found the N-terminal amino group in lysozyme not to be fructosylated (*28*).

On the other hand, MALDI-TOF-MS was also effective as a rapid means to monitor the rate of glycation by the collection of MS spectra obtained at different incubation times. For example, the component at *m*/*z* 1196.1 was not observed in LA−Fru and LA−Psi conjugates incubated for 12 h (**Figure 4**), but it was detected in conjugates after 24 h of incubation (not shown), suggesting that Lys_{114} was slowly glycated with ketohexose. In addition, the binding sites on Lys_{13} in all four LA−sugar conjugates and on Lys_{122} in two LA−aldose conjugates for 24 h of incubation were also detected (**Table 1**). The results were in good agreement with the time−course study on LA glycation described above.

One of the most important areas in food analysis is the detection, characterization, and localization of modifications on peptide sequences, which occur during processing of food components. Particularly, the localization of these modifications in protein sequence is of special interest because they affect the functional as well as nutritional properties of proteins and derived peptides. A great deal of studies has focused on site specific quantitative evaluation of the glycated protein with different sugars by using mass spectrometry, such as glycated egg white lysozyme with Glc (*25*, *28*) and glycated bovine β-lactoglobulin with lactose (*29*). However, this study is the first to evaluate the primary structural modification of protein with rare sugar. It could be very important to understand the relationship between the glycation reaction in the early stage

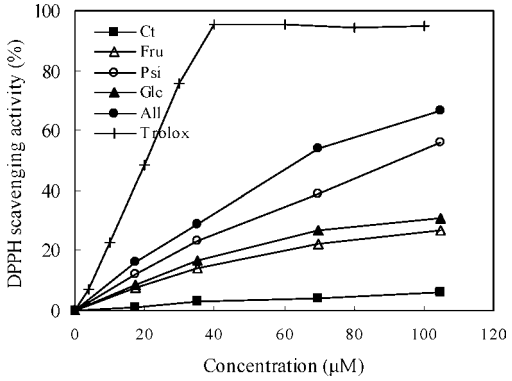

Figure 5. Effects of concentration of Trolox and glycated samples on DPPH radical scavenging activity. The control (Ct) and modified LA with four reducing sugars (Fru, Psi, Glc, and All) were incubated at 50 °C and 55% RH for 48 h. Trolox as the standard gives a linear response (R^2 = 0.997) in the concentration range of 0–40 μM. All results were determined at least three times and in triplicate.

and the functional properties of MRPs formed from the advanced stage of MR.

DPPH Radical Scavenging Activity of Glycated Proteins/Peptides. The DPPH radical is a convenient reagent for the radical scavenging assay and has been proven to be absorptive at 517 nm (*11*). DPPH has the advantage of being independent of any enzyme and free of additive-induced complications such as enzyme inhibition. In this experiment, control and glycated LA for 48 h of incubation and their peptic hydrolysates were determined for their free radical scavenging activity by using DPPH method.

As shown in **Figure 5**, the concentration-dependent curve was first plotted as the percentage of DPPH radical scavenging activity. The results showed that radical scavenging activity of control LA was 5.8% in a higher concentration of 105 μM (1.5 mg/mL), showing a weak radical scavenging activity of nonglycated LA. In contrast, LA glycated with Psi and All showed the higher radical scavenging activity depicted by about ~56.3 and ~66.9%, and LA modified with Fru and Glc showed ~26.5 and ~36.8% of radical scavenging activity, respectively. These results indicated that glycation could induce a radical scavenging activity to LA, and the intensity of which depended on the reducing sugar used for glycation. Furthermore, to test the effect of free sugars in glycated samples, the antiradical activities of four sugar solutions at the range of concentration from 0.1 to 1 mg/mL were also determined by using the DPPH method. The result showed that all four sugars did not exhibit any free radical scavenging activity in this concentration range (data not shown).

On the other hand, the increase in scavenging activity in Trolox standard curve was linearly related to the increase in the concentration of Trolox in the range from 0 to 40 μM, and then, a plateau was rapidly attained. In the case of glycated LA, it was shown that the scavenging activity increased significantly within a protein concentration of 70 μM and then appeared to increase more slowly than a protein concentration of 70 μM, except LA−Psi conjugates. Thus, 70 μM (1 mg/mL) protein concentration was selected as a function of concentration in order to compare the nonhydrolyzed samples and their hydrolysates.

To test whether hydrolysis has a resistant effect on the antioxidant activity, the protein−sugar conjugates were digested and the free radical scavenging activity of hydrolysates was investigated. As preliminary experiments have shown, the proteolytic capability of pepsin toward the nonreduced LA was much higher than that of V8 protease, trypsin, and chymotrypsin.

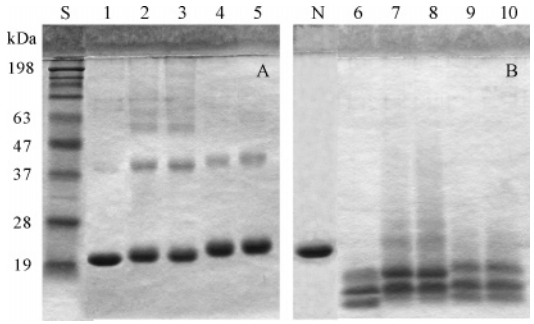

Figure 6. SDS–PAGE profiles of glycated LA samples (**A**) and their peptic hydrolysates (**B**). S, standard protein; N, native LA. Glycated LA with the following: 1, no sugar; 2, Fru; 3, Psi; 4, Glc; and 5, All. Peptic hydrolysates from the following: 6, control LA; 7, LA-Fru; 8, LA-Psi; 9, LA-Glc; and 10, LA-All. Protein was incubated with four reducing sugars at 50 °C and 55% RH for 48 h.

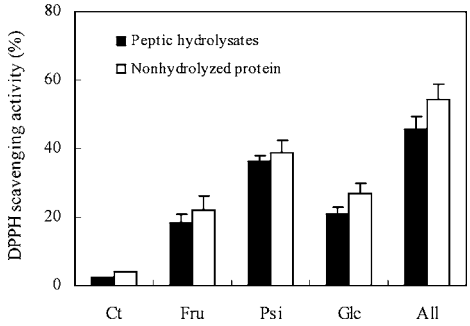

Figure 7. DPPH radical scavenging activity of nonhydrolyzed protein samples and their peptic hydrolysates. The control (Ct) and glycated LA with four reducing sugars (Fru, Psi, Glc, and All) were incubated at 50 °C and 55% RH for 48 h. Data shown are mean values ± SD of three complete sets of experiments (n = 9).

Pepsin thus turned out to be the most suitable enzyme for the digestion of LA in nonreduced form. SDS−PAGE profiles showed that a control sample in acid state was digested completely by pepsin (**Figure 6B**). Although glycated samples showed a little resistance to peptic hydrolysis, the monomeric and dimeric bands of samples almost disappeared, followed by the appearance of two or three broad bands. MALDI-TOF-MS spectra also showed that control and glycated LA proteins were hydrolyzed in a mass range from m/z 500 to 6000 by pepsin (data not shown). Dolgikh et al. (*30*) reported that the acid state of LA at low pH values is a classical molten globule state. In the case, a subset of hydrophobic residues is easily exposed to a solvent accessible environment (*31*). Pepsin has a broad substrate specificity, cleaving at the C terminus of bulky and hydrophobic amino acid residues at a lower pH value. Therefore, LA could be rapidly hydrolyzed by pepsin due to a molten globule state.

As shown in **Figure 7**, the antiradical property of peptic hydrolysates was investigated in different glycated LA samples to assess the influence of hydrolysis on radical scavenging activity. The results showed that the radical scavenging activities of peptic hydrolysates all decreased as compared to those of corresponding nonhydrolyzed samples. The differences were about 2.2, 3.8, 6.4, and 8.5% for LA modified with Psi, Fru, Glc, and All, respectively. Nevertheless, even if the radical scavenging activity decreased after peptic hydrolysis of glycated LA, such an activity of glycated LA with rare sugars was still higher as compared to that of LA modified with control sugars. As shown in t-test results, all of the sugar−protein complexes and their hydrolysates increased significantly ($p < 0.05$)

antiradical activity as compared to Ct samples. Furthermore, significant differences were also found among four complexes and their hydrolysates ($p < 0.05$), and the order of their antiradical activities was as follows: All→ Psi→ Glc→ Fru− proteins/peptides. Thus, the glycated food proteins with rare sugars could provide a large source of antioxidant peptides to help strengthen the radical scavenging effect of the organism.

The reducing sugar reacts nonenzymatically with ϵ-amino groups of exposed Lys residues belonging to a protein chain: After the reducing sugar addition, extensive dehydration/oxidation reactions take place, leading to a highly reactive species, which could explain the different functionality and reactivity exhibited by glycated protein. However, in the present study, it was found that the free radical scavenging activity of glycated LA was not directly related to the glycation degree and position. It has been reported that the extent of conjugated proteins with two aldoses (Glc and All) or two ketoses (Fru and Psi) was very similar, but their cross-linking and browning/fluorescence intensity showed a significant difference (*5*, *6*, *19*). The results suggested that these chemical properties of MRPs might be related to the rate of conversion of Amadori rearrangement to chromo/fluorophoric adducts, while they may not be related to the rate of formation of the initial Schiff base adducts. Nishino et al. (*32*) reported that formation of heterocycles in the advanced stage of MR could explain the ability of glycated proteins to react with radical compounds. We speculated that the formation of diversified MRPs could be induced by structural differences between sugars, and subsequent forming products might contribute to the functional properties of MRPs.

In conclusion, MALDI-TOF-MS spectrometry has been successfully applied in the analysis of nonenzymatic glycation of LA. By comparing with peptide mapping in the range of mass from *m*/*z* 500 to 3500, originating from protease V8 digestion of LA, the glycated peptides and the binding sites of hexose to LA were identified. Seven potential glycation sites were located on Lys_{13}, Lys_{16}, $Lys_{93/94}$, Lys_{98}, Lys_{108}, Lys_{114}, and Lys_{122} for protein−aldose conjugates and three sites on Lys_{13}, $Lys_{98/108}$, and Lys_{114} for protein−ketose conjugates. The results showed that although there was a greater propensity for Maillard-induced attachment of aldo-hexose than keto-hexose, there was not a marked difference between rare sugar and corresponding control sugar for the glycation degree and position. On the other hand, the glycated proteins/peptides with rare sugars were more effective in scavenging free radicals than those modified with control sugars. From these results, it was inferred that free radical scavenging properties of glycated protein/peptide were not directly relative to their primary structural modifications in the early stage, while the formation of heterocycles in the advanced stage of the MR induced by different reducing sugars might contribute to the radical scavenging properties of MRPs.

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