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Modification of Ovalbumin with a Rare Ketohexose through the Maillard Reaction: Effect on Protein Structure and Gel Properties

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The effect of nonenzymatic glycation on the structural changes and gelling properties of hen ovalbumin (OVA) through the Maillard reaction was studied. OVA was incubated at the dry state with a rare ketohexose (D-psicose, Psi) and two alimentary sugars (D-fructose, Fru; D-glucose, Glc) at 55 °C and 65% relative humidity. To evaluate the modification of OVA by different reducing sugars during the glycation process, the extent of the Maillard reaction, aggregation processes, structural changes, and gelling behaviors were investigated. Reactivity of Psi with the protein amino groups was much lower than that of both Fru and Glc, whereas Psi induced production of browning and fluorescent substances more strongly than the two alimentary sugars did. Furthermore, OVA showed an increased tendency toward multimeric aggregation upon modifying with Psi through covalent bond. The modified OVAs with reducing sugar were similar to nonglycated control sample in Fourier transform infrared (FT-IR) characteristics, but significantly decreased in intensity of tryptophan-related fluorescence. The results indicate that although glycation brought about similar changes in the secondary structure without great disruption of native structure, its influence on the side chains of protein in tertiary structure could be different. Breaking strength of heat-induced glycated OVA gels with Psi was markedly enhanced by the Maillard reaction. These results suggest that Psi had a strong cross-linking activity with OVA; consequently, the glycated OVA with Psi could improve gelling properties under certain controlled conditions.

KEYWORDS: Ovalbumin; D-psicose; glycation; Maillard reaction; gelling property

INTRODUCTION

The nonenzymatic interaction between reducing sugars with amino groups of the lysine residue of proteins, known generally as the Maillard reaction, has proven to be extremely important in food science. The interaction comprises a complex network of reactions, which is known to result in the formation of both large protein aggregates and low molecular weight products that are believed to impart the various flavor, aroma, and color characteristics found in foods. Over the past few years there has been growing interest in the effect of reducing sugars on protein structural functionality in compositionally complex food systems (*1*−*3*). It has been reported that the glycated proteins could improve the functional properties of food, such as thermal stability, emulsifying ability, foaming properties (*4*−*6*), antioxidative activity (*7*), and gelling properties (*8*, *9*).

Egg white protein (EW) is extensively utilized as a functional food material in food processing. A major functional property of EW is to improve the consistency of foods by forming thermally induced gels. Matsudomi et al. (*8*) reported that a transparent and firm gel could be made from dried EW modified with galactomannan through the Maillard reaction. Handa and Kuroda (*10*) showed that Maillard products of dried EW containing glucose enhanced the heat-induced gelling properties, such as gel strength and water-holding capacity. EW proteins modified by the Maillard reaction play a major role in controlling the structure, texture, and stability of food colloids through their gelling and aggregation behaviors (*9*). However, the mechanisms responsible for the significant improvement of gelling properties of Maillard products of dried EW proteins are not fully explained because of the complicated interactions among the different proteins. Ovalbumin (OVA) is the main constituent of EW proteins; its behavior predominantly affects the gelation of EW (*11*). Therefore, monitoring the physicochemical and structural changes of Maillard-reacted OVA will help to further the understanding of the relationship between the structure and gelling properties of Maillard products of dried EW.

Rare sugars are defined as "monosaccharides and their derivatives that are rare in nature". D-Psicose (Psi), a rare ketohexose, is present in small quantities in commercial mixtures of D-glucose (Glc) and D-fructose (Fru) obtained from the hydrolysis of sucrose or isomerization of Glc (*12*, *13*). In past decades,

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some isomers of monosaccharides, for example, L-sugar or D-tagatose, have been developed as alternative carbohydrate sweeteners and bulking agents (*14*, *15*). However, Psi has not yet been studied as a sugar substitute, because it is not abundant in nature and is difficult to prepare by chemical methods. Recently, an improved method (*16*) has appeared to be applicable to the large-scale production of Psi from Fru, which should allow commercial utilization of this expensive sugar. On the other hand, Matsuo et al. (*13*, *17*) have reported that Psi supplements in diets suppressed hepatic lipogenic enzyme activity and provided zero energy. Therefore, Psi might be useful in the food industry as a noncalorie sweetener for obese people as an aid for weight reduction. However, it was not known whether the structural and functional properties of the glycated protein with Psi were identical with or distinct from those of glycated protein with alimentary sugars. To contribute to the understanding of the structure−function relationship, we focused our attention on the structural and physicochemical changes, as well as the gelling properties of glycated OVA with a rare sugar (Psi), and two alimentary sugars (Fru and Glc) controlled as keto- and aldohexose, respectively.

Although many studies indicated the functional improvements of proteins by conjugation with saccharides, structural and physicochemical studies of the conjugates are still inadequate. The objective of the present study is to investigate the physicochemical and conformational changes, as well as the gelling properties of the glycated OVA with Psi through the Maillard reaction by comparison with alimentary sugars. The reactivity of reducing sugars, with respect to their ability to utilize primary amino groups of proteins, to cross-link proteins, to develop Maillard fluorescence and browning, and to reduce protein solubility were investigated. At the same time, the structural changes and the gelling behaviors of glycated OVAs were also characterized. Attempts would be made to deepen the understanding of the molecular mechanisms underlying a change in conformation, physicochemical properties, and gel formation of Psi-glycated proteins.

MATERIALS AND METHODS

Materials. OVA was purified from fresh hen egg white by a crystallization method in an ammonium sulfate solution and recrystallyzation three times as described by Sun and Hayakawa (*18*). D-Psicose (Psi) was obtained from Kagawa Rare Sugar Cluster (lot 010629, 3, 044). d-Glucose (Glc), D-fructose (Fru), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 8-anilino-1-naphthalenesulfonic acid (ANS), and trinitrobenzenesulfonic acid (TNBS) were purchased from Wako Pure Chemical Industries, Ltd. All other chemicals used in this study were of analytical grade.

Glycation of OVA Protein. Glycation was performed with a rare sugar (Psi) and two alimentary sugars (Fru and Glc), respectively. OVA was dissolved in 20 mM carbonate buffer (pH 9.0) at a protein concentration of 5% (w/v) with the sugars (8% of the protein dry weight), that is, a molar ratio of about 1:20 of protein versus sugar. OVA contains 20 amino groups of lysine residues and one acetylated terminal amino group. OVA−sugar solutions were lyophilized. The dried samples were incubated at 55 °C and 65% relative humidity using a saturated KI solution for 1−4 days. A mild heating (55 °C) was chosen to limit thermal denaturation and aggregation effects, and this temperature is also often used in accelerated storage trials. For these procedures, control experiments were carried out with no added sugars. Incubations were set up in triplicate in individual vials, and all assays were done in triplicate. Glycated proteins were dialyzed overnight at 4 °C to remove free sugars, against distilled water, lyophilized, and then kept at −20 °C until use.

Measurement of the Extent of Glycation. The brown color of glycated OVA (10 mg/mL) was analyzed by measuring the absorbance at 420 nm. Fluorescence emission spectra were determined on glycated OVA, which had been incubated for 2 days. The sample was dissolved in 10 mM phosphate buffer (PB, pH 7.0) at a protein concentration of 1 mg/mL. The fluorescence intensity (FI) of glycated OVA was measured in triplicate, using an F-2500 spectrofluorometer (Hitachi Co.). The excitation and emission wavelengths were 350 and 415 nm, respectively. FI was expressed as relative fluorescence intensity in arbitrary units (AU).

The free amino groups were measured according to the TNBS method (*19*). To 1 mL of protein solution (0.8 mg/mL) was added 1 mL of 4% $NaHCO_3$ and 1 mL of 0.1% TNBS in water. The solution was incubated at 40 °C for 2 h. Then 1 mL of 10% sodium dodecyl sulfate was added, followed by 0.5 mL of 1 N HCl. The absorbance of the solution, diluted 2-fold with 0.01 N HCl, was read at 340 nm against a blank treated as above but containing 1 mL of water instead of the protein solution. The wavelength was selected as 340 nm, at which the solution had maximum absorbance in a UVmini-1240 spectrophotometer (Shimadzu Co.).

Measurement of Solubility. The glycated samples were dissolved in distilled water at a protein concentration of 1% (w/v) and then centrifuged at 5000*g* for 10 min. The supernatant was stored at 4 °C until used. Solubility was measured by determining the protein concentration in the supernatant according to the method of Lowry et al. (*20*), using bovine serum albumin as a standard. Protein solubility was represented as a percentage of protein content of Maillard-reacted sample to nonglycated sample.

Gel Electrophoresis. Maillard-reacted OVA samples were resolved in 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue, according to the method of Laemmli (*21*). Electrophoresis was done in both reducing and nonreducing conditions in the presence and absence of 2-mercaptethanol (2-ME), respectively. To assess the structural changes of glycated OVA samples, native PAGE without SDS and reducing agent was also performed with a 10% polyacrylamide gel sheet.

Measurement of Sulfhydryl Thiol (SH) Groups. Free SH group content was determined using DTNB ′ according to the method of Ellman (*22*). Absorbance was read at 412 nm. The content of SH groups was calculated from nine measurements of three separate experiments using a molar extinction coefficient of 13600 M^{-1} cm^{-1}.

Measurement of Surface Hydrophobicity (S_0) and Tryptophan (Trp)-Related Fluorescence. The samples were dissolved in 10 mM PB (pH 7.0) to give 1.0% (w/v) OVA concentration and then diluted with the same buffer for a series of five concentrations between 0.1 and 0.5%. The S_0 of a treated sample was determined according to the fluorescence probe ANS method of Hayakawa and Nakai (*23*). A sample with no added ANS was used as a control because the glycated samples generated some fluorescent substances. FI was measured at an excitation wavelength of 390 nm and an emission wavelength of 470 nm. For each determination, a 0.04 mM ANS methanolic solution was used to adjust the relative FI. The initial slope of the FI versus protein concentration (percent) plot, which was calculated by linear regression analysis, was used as an index of the protein S_0. Trp-related fluorescence (excitation at 280 nm) was measured by using 0.3 mg/mL protein solutions in 10 mM PB (pH 7.0).

FT-IR Spectroscopic Analysis. The secondary structural changes of incubated sample (5%) were analyzed by FT-IR spectroscopy. An FT-IR 670 Plus spectrometer (JASCO Co.) was used to determine the IR spectra of the glycated and control samples previously deposited on infrared-transparent CaF_2 windows. The FT-IR spectra were collected at a resolution of 4 cm^{-1} and 32 scans. All IR spectra were baseline corrected and area normalized between 1600 and 1700 cm^{-1} using the Windows software supplied (JASCO). The content of secondary structure was determined from the average of three replicate spectra against a blank background.

Preparation of Gel and Breaking Test. The protein samples were dissolved in 86 mM NaCl at a protein concentration of 8% (w/v) and adjusted to pH 8.0 with 1 N NaOH. In previous investigations (*11*), it was shown that at alkaline pH values (pH 8.0) OVA formed reasonably well cross-linked networks in which rheological properties could be readily assessed. The sample solution was degassed under vacuum for 1 h. An aliquot of 5 mL of sample solution was put into a small Petri

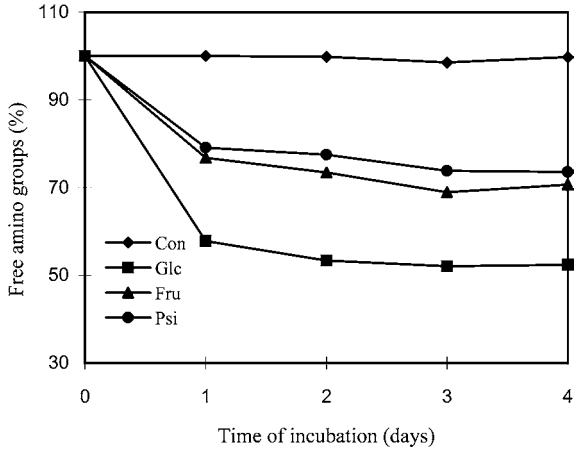

Figure 1. Changes in free amino group contents of glycated OVA with reducing sugars incubated for 1–4 days. Data represent the mean value of the nine determinations from different samples set up in triplicate in individual vials, with a standard deviation of <2%. The mixtures of OVA with reducing sugars (molar ratio; OVA/sugar = 1:20) were incubated at 55 °C and 65% relative humidity.

dish (diameter = 22 mm) covered with a silicone plate and a glass plate, heated at 80 °C for 30 min in a water bath, followed by cooling in tap water for 15 min, and equilibrated to ambient temperature for 15 min. The gelling properties were measured by using a Rheoner II creep meter (RE 2-3305, Yamaden Co.) with a 2 kgf load cell. The gel sample prepared in a small Petri dish was placed on the stage of the rheometer, and the breaking stress and breaking strain were measured by the constant-speed compressive breaking test (compression rate = 1 mm/s) by means of a 0.3 cm diameter rob plunger. The breaking strength was measured and read on the stress versus strain curve by the value of the first force peak. Breaking stress of gels was calculated from the load value at breaking point divided by the initial cross-sectional area of the plunger. Breaking strain was determined as the ratio of the deformation at a breaking point to the initial height. The breaking strength was measured five times on each dish, and each sample was measured in at least eight dishes. Data represent the mean values of 40 measurements, and standard deviation was calculated.

RESULTS

Determination of Free Amino Groups. At an early stage of the Maillard reaction, the protein containing free amino groups, such as the ϵ-NH_2 groups of lysine and arginine, reacted with carbonyl groups of sugars to cause the loss of free amino groups (*24*). OVA was incubated with various sugars for 1−4 days, and the loss of free amino groups in glycated OVA was analyzed by using the TNBS method. The result showed that the content of free amino groups in glycated OVAs significantly decreased after 1 day of incubation and then displayed less change after 2 days of incubation (**Figure 1**). The loss of free amino groups in Glc-glycated OVA was higher than that of Fru/Psi-modified OVAs, whereas this loss in OVA alone was not observed throughout the incubation for 4 days. The terminal α-amino group of OVA is acetylated; thus, all free amino groups of OVA originate from the ϵ-amino groups of 20 lysine residues. The numbers of modified amino groups were calculated from the percent of modified amino groups and the number of total amino groups. The average number of sugars linked per OVA molecule by amino groups reached about 9.3, 5.3, and 4.5 for Glc, Fru, and Psi, respectively, after 2 days of incubation, suggesting that Glc showed a higher initial rate of utilization of primary amino groups than Fru/Psi.

Assessment of Brown Color Development. Glycation was accompanied by a yellow coloration, whereas the controls

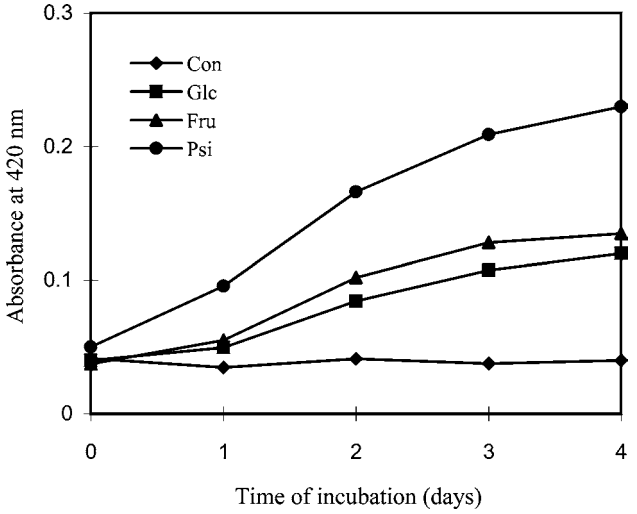

Figure 2. Changes in browning of OVA during 4 days of incubation with reducing sugars. Data shown are the mean value of triple determinations, with a standard deviation of <0.01 absorbance unit. Nonenzymatic browning was determined at 420 nm, protein concentration of 1%. The glycated OVA was prepared as described in **Figure 1**.

remained colorless. The 420 nm absorption spectra obtained for Glc−, Fru−, and Psi−OVA samples during 4 days of incubation are shown in **Figure 2**. With an increase in incubation time, absorbance at 420 nm also increased in all solutions of sugar−protein. Clearly, a large difference in 420 nm absorbance readings between Psi−OVA and Fru−/Glu−OVA solutions was observed throughout incubation time. In the present study, the rate of nonenzymatic browning in the Psi-glycated OVA was found to be the highest among three sugars. The Fru-glycated OVA browned at the second fastest rate, but its rate was closer to the rate of the Glc-glycated OVA than that of Psi-glycated protein.

Fluorescent Substances. The fluorescence spectra (excitation at 350 nm) of glycated OVAs with various reducing sugars were quite different (data not shown). Glc was indicated to be relatively inactive in generating fluorescent substances, and Fru was found to have a slightly greater reactivity, whereas Psi had a particularly high fluorescent reactivity with OVA. At the same time, it was observed that maximum FI was yielded at an emission wavelength of 415 nm in the fluorescence spectra. The result indicated that Psi−OVA yielded very high fluorescence intensity (50 AU), which was about twice that of Glc (26 AU), and Fru−OVA displayed intermediate FI (39 AU) after 2 days of incubation (**Table 1**). No fluorescence in incubated OVA alone was observed for 2 days of incubation.

Solubility of Glycated OVAs. In general, solubility is an index of heat denaturation of proteins. Data for the solubility of glycated OVA with different reducing sugars (**Figure 3**) showed that the samples incubated within 2 days were almost soluble in water. However, after incubating for 3 days, the solubility of glycated OVAs decreased gradually with increasing incubation time; especially, a significant loss of ∼21% of solubility was observed in the glycated OVA with Psi incubated for 4 days. The result showed that Psi-modified OVA could have a higher cross-linked reaction and subsequently cause a higher denaturation/aggregation of protein. From these results, the glycated OVAs with various reducing sugars incubated for 2 days were used for the following experiments, and the incubated OVA alone for 2 days was used as a control in all measurements.

Table 1. Changes in Secondary Structure, Surface Hydrophobicity (S_0), SH Groups, and Fluorescence Intensity (FI) of Glycated OVA after 2 Days of Incubation

sample	content of secondary structure (%)[a] α-helix	β-sheet	S_0[b]	content of SH groups (μmol/g OVA)[c]	FI[d] (AU/mg)
native OVA	24.2	31.2	5.1	81.2	0
Ct–OVA	23.0	34.0	9.6	72.2	0
Glc–OVA	22.3	35.5	8.1	68.4	26
Fru–OVA	22.3	35.3	8.3	66.4	39
Psi–OVA	22.6	35.0	7.8	62.7	50

[a] Standard deviations for α-helix and β-sheet were all <0.6%, and there were no significant differences between the control and glycated samples ($p > 0.05$). [b] S_0 was expressed as an initial slope of the FI vs protein concentration plot, with standard deviations of <0.01. [c] Standard deviations for SH groups were all <1.36 μmol/g of protein. [d] Fluorescence was expressed as the increase in ex 350/em 415 fluorescence compared to control proteins, which remained unchanged during the incubation. The results are expressed as arbitrary fluorescence units (AU).

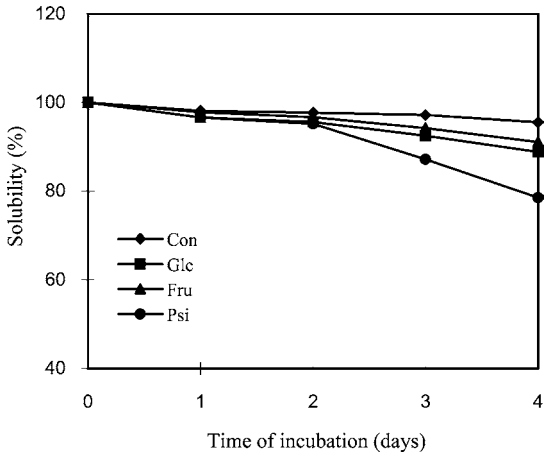

Figure 3. Solubility of glycated OVA with different reducing sugars, as a percentage of protein content of Maillard-reacted sample to nonglycated sample. Protein content in the supernatant was determined according to the Lowry method. All solubility determinations were done in triplicate. The standard deviations were all <1.6%. The glycated OVA was prepared as described in **Figure 1**.

Sulfhydryl (SH) Group Oxidation. The native OVA protein used in the present measurement has an SH group content of 81.2 μmol/g of OVA, ~4 SH groups. **Table 1** shows that the incubation without reduced sugars slightly interferes with the redox state of protein SH groups after incubation for 2 days. The SH group content in glycated OVAs significantly decreased, following the order Glc > Fru > Psi. The data suggested that SH groups were employed in protein cross-linking by SS bonds. On the other hand, glycation with different reducing sugars might cause changes in partial conformation of protein and lead to pronounced disturbances in SH group oxidation state.

Aggregation Process. SDS-PAGE analysis of glycated OVA in the presence (**Figure 4A**) and absence (**Figure 4B**) of 2-ME, respectively, was performed. As expected, a large amount of high molecular weight aggregates was observed in glycated OVA compared with nonglycated OVA under reducing and nonreducing conditions (**Figure 4A**,**B**, lanes 2, 3 and 4), especially when glycation was performed with Psi (lane 4). In the presence of ME (**Figure 4A**), dimer bands almost disappeared in all incubated samples. The density of monomer and oligomer bands significantly decreased with Psi-glycated OVA, whereas the density of high molecular weight aggregates band not entering the separating gel increased (**Figure 4A**, lane 4).

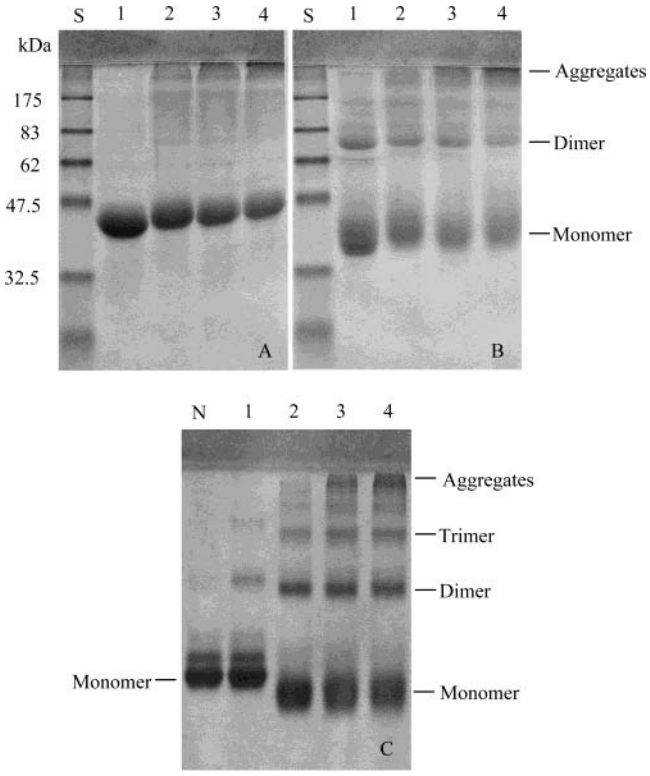

Figure 4. Electrophoretic profiles of native and glycated protein: (**A**) SDS-PAGE (12.5%) with ME; (**B**) without ME; (**C**) native PAGE (10%) profile. OVAs were incubated with and without reducing sugars for 2 days. S, standard protein; N, native. Two-day incubations with (lane 1) no sugar, (lane 2) Glc, (lane 3) Fru, and (lane 4) Psi.

On the other hand, as shown in SDS-PAGE profiles without ME (**Figure 4B**), incubation led to the appearance of disulfide dimers for all incubated samples. The results suggested that covalent bonds, both sugar−lysine amino carbonyl and intermolecular SS bonds, were involved in the polymerization. From native PAGE profiles (**Figure 4C**) of incubated sugar−OVA it appeared that the monomer OVA bands moved to the anode (lanes 2, 3, and 4), indicating that the reduced sugars attached to amino groups of OVA and decreased positive charges on the protein surface.

In addition, the effect of three reducing sugars on the cross-linking of OVA was compared by SDS-PAGE analysis throughout incubation for 1−4 days (data not shown). The result showed that both rate and extent of cross-linking of OVA by Psi were significantly greater than that by Glc/Fru, suggesting a greater tendency for aggregation through covalent cross-linking of Psi−protein.

FT-IR Spectroscopic Analysis. Table 1 shows the changes in secondary structure of the glycated and unmodified protein by FT-IR spectroscopy. The glycated OVAs had slightly lost α-helical structure with a concomitant increase in β-sheet proportion, but were similar to incubated OVA alone. Such changes were assumed to be due to incubation at 55 °C and occur along with a slight unfolding in secondary structure, rather than modifying by reducing sugar. In addition, the OVAs modified by different reducing sugars were not seen to have significant differences in their FT-IR characteristics ($p > 0.05$), indicating that no correlation was observed between the extent of glycation and the secondary structural change.

Surface Hydrophobicity (S_0). The effect of the glycation treatment on the conformational state of the modified OVA was evaluated by S_0 using the ANS binding method. The results

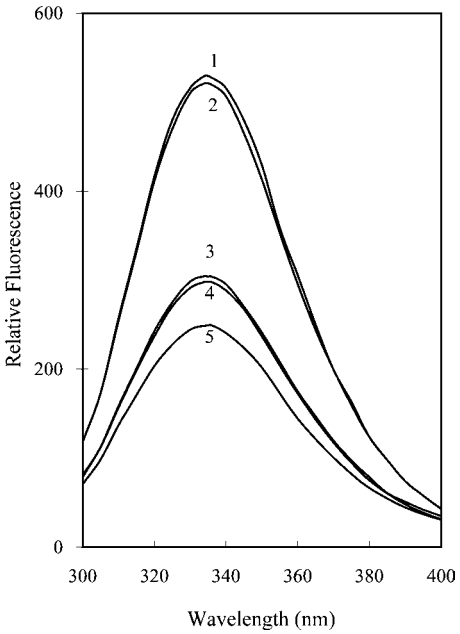

Figure 5. Tryptophan fluorescence spectra of glycated OVA. The excitation wavelength was 280 nm, and the emission was scanned from 300 to 400 nm. Sample was incubated for 2 days as described in **Figure 1**. Fluorescence spectra of samples were measured at 0.3 mg/mL in triplicate. 1, Native OVA; two-day incubation with (2) no sugar, (3) Glc, (4) Fru, and (5) Psi.

showed that the S_0 of glycated OVAs incubated for 2 days was found to have a slight increase as compared with native OVA (**Table 1**). This effect could be attributed to the incubation treatment, because there were no significant differences between the control and glycated samples ($p > 0.05$). Such a result indicated that the glycated OVA with reducing sugar could not expose newly hydrophobic patches on the protein surface, suggesting that glycation did not significantly affect the structural state of OVA.

Tryptophan Fluorescence. Trp fluorescence is a sensitive index of alteration in protein conformation and amino acid loss. The incubated OVA alone was found to have no alteration in Trp-FI compared with native OVA. However, changes in Trp-related fluorescence were observed for all glycated proteins by fluorescence emission spectra after excitation at 280 nm (**Figure 5**). The strongest reduction was observed for Psi-modified protein (46.4% of the initial fluorescence measured for the control OVA). Interestingly, Trp-related fluorescence in Fru-glycated OVA was similar to that obtained in Glc-glycated OVA. In the present study, a reduction in intensity of Trp-related fluorescence was observed in glycated proteins with all three reducing sugars, suggesting that the glycation affected partially the side chains of protein in tertiary structure through the Maillard reaction without great disruption of native structure.

Gelling Properties. Heat-induced gelation is one of protein's important functional properties with respect to EW usage in food systems. In this study, gel properties of OVA alone and of glycated OVA with various reducing sugars were investigated by measuring breaking stress and strain and analyzing visual appearance. The native OVA gel was turbid, had a soft texture, and exhibited considerable syneresis. Compared with native OVA gel, the gel strength of control OVA gel (incubated for 2 days without sugar) did not show a significant difference ($p > 0.05$) (**Figure 6**), and its appearance remained turbid. The breaking stress and strain for all glycated OVA gels increased greatly, and the appearance of these gels was transparent. On

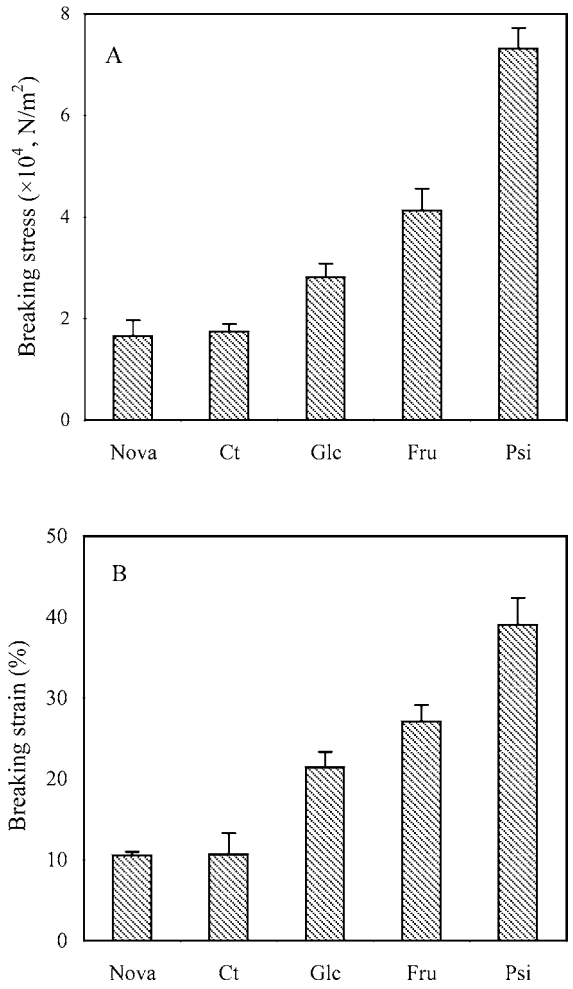

Figure 6. Breaking stress (**A**) and breaking strain (**B**) of glycated OVA gels with reducing sugars through Maillard reaction. Gels were produced by heating 8% (w/v) sample solutions (containing 86 mM NaCl, pH 8.0) at 80 °C for 30 min. Data represent the mean of 40 times from eight replicates. Error bars show standard deviation of means.

the other hand, comparison of the gel properties among the three reducing sugars showed that the gel strength of glycated OVA gel with Psi was significantly increased through the Maillard reaction ($p < 0.01$), the transparency was also highest, and these gel properties were of the order Psi > Fru > Glc. Although the mechanism of gelling formation for glycated OVA with Psi was not clear, this certifies that the effects of glycation with Psi appeared significantly to be improved gelling properties in comparison with protein modified with Fru and Glc through the Maillard reaction.

DISCUSSION

Glycation of proteins and their subsequent structural and functional modifications have been ascribed to play a prominent role in food science. This investigation has been conducted to explore the effect of glycation on structural and functional properties by use of Psi and control sugars (Fru and Glc). Glc and Fru were selected as control sugar reactants due to the difference in aldose and ketose structure and the fact that each sugar produces different amounts and types of the Maillard reaction products (*25*, *26*). In general, aldoses are considered to be more reactive than ketoses because of their more electrophilic carbonyl groups (*27*, *28*). However, ketose sugar Fru is found to brown more quickly than the aldose isomer Glc,

because it has a high concentration of acyclic forms in aqueous solutions (*24*, *29*, *30*). Our results also showed that the reactivity of Glc with the protein amino groups was higher compared with Fru. In contrast, Fru was more reactive in browning, fluorescence, and protein cross-linking than Glc in the same reaction conditions. On the other hand, although OVA glycated with a rare ketose, Psi, in some physicochemical properties was closer to Fru-glycated protein, the cross-linking activity, the browning, and fluorescence intensity of Psi-glycated OVA were quite higher compared to protein glycated with either Fru or Glc, suggesting that Psi might display strong cross-linking chemistry.

In an attempt to shed more light on the relationship between the glycation process and protein structural alterations, we explored the structural change of the glycated OVA by different methods. The results indicated that OVA glycated with different reducing sugars did not bring about a significant change compared with control protein by ANS-binding and FT-IR spectral measurement, suggesting that the glycated molecules have a higher degree of secondary structural order. Trp fluorescence intensity of modified OVA was found to have a significant decrease upon glycation, whereas the fluorescence intensity between incubated and nonincubated OVA alone showed no change, implying that the side chains of protein (tertiary structure) were partially altered by glycation with reducing sugars. Other studies had also indicated tertiary structural alteration in protein upon glycation (*31*). On the other hand, some recent investigations have demonstrated that denaturation of many globular proteins proceeds via a state called a molten globule (*32*). The molten globule state is a unique state in which the protein molecule has a native-like backbone secondary structure, whereas the side chain's environment undergoes a denaturation-like alteration; therefore, the protein molecule is somewhat expanded but retains a more compact conformation compared with the fully denatured state. The molten globule state has been shown to be involved in the functional properties of food proteins (*33*). The fact that the glycation of OVA hardly affected the secondary structure also suggested the formation of the molten structure in glycated OVA.

Cross-linking studies showed that the OVA glycated with Psi resulted in protein cross-linking at a considerably faster rate, and OVA was easily polymerized by cross-linking through Psi−lysine amino carbonyl and intermolecular SS bond adducts. However, the extent of lysine loss was not very serious, reflecting the lower reactivity of Psi with protein through the Maillard reaction. In addition, the sudden loss of lysine for Glc-glycated OVA was not accompanied by a sudden increase in cross-linking. Differences in the rate of amino−carbonyl reaction, as have been observed between OVA and different reducing sugars, probably do not affect the overall kinetics. The results suggested that the initial reaction of primary amino groups with reducing sugar might not be the rate-determining step of the cross-linking process, which might therefore reflect subsequent chemistry.

The Maillard reaction sequence is initiated by the reversible formation of a Schiff base, which undergoes an Amadori rearrangement to yield a relatively stable ketoamine adduct during early glycation. A series of further reactions form a variety of structurally diverse compounds known as advanced glycation end products (AGEs), which frequently have chromophores, fluorophores, and protein cross-links (*34*). The chemical and functional modifications are produced in the late stages. Our results have indicated that Glc-glycated OVA was relatively slow to generate fluorescent substance and aggregates, suggesting that the formation of compounds which might not contribute to protein crossing-linking, such as N^{ϵ}-carboxymethyllysine (CML), might have occurred. CML, a colorless and nonfluorescent compound, is unlikely to lead to the formation of multimeric protein, because it does not contain either a free amine or a reactive carbonyl group (*35*, *36*). It has been indicated that autoxidative cleavage of the Schiff base or Amadori adducts to protein has been found to generate CML (*37*). However, glycation with Psi appeared to have particularly high fluorescent reactivity and to induce a greater tendency for aggregation in the OVA by the intermolecular cross-linking. This might be explained by the fact that Psi−protein adducts, which had undergone less oxidative cleavage, were available for further reaction, either with unreacted protein monomer or with other protein adducts. Subsequently, they were able to enhance cross-linking and fluorescent reaction. In brief, OVA glycated with Psi can generate a higher browning and cross-linking reaction by a faster conversion of its Amadori groups without oxidative cleavage, further improving its gelation property.

In addition, Fru and Psi are epimers with respect to C3, differing only by configuration about one C atom. The aggregation and gelling behaviors were significantly different, suggesting the configuration about carbon 3 might play a role in the cross-linking activity. However, it was structurally unclear how the configuration of the hydroxyl group (OH) at carbon 3 could so markedly increase the cross-linking activity. Protein browning, fluorescence, and cross-linking are characteristic features of the Maillard reaction. In this study, although the physicochemical properties and cross-linking ability of Psi with protein were well-documented, the mechanism of reaction is still not clearly established, requiring further systematic investigations.

In conclusion, our results demonstrated that glycation with three reducing sugars could not significantly alter the secondary structure of the OVA, whereas it could lead to partial conformational change of glycoforms with respect to the side chains of protein in tertiary structure. The result indicated that the OVA glycated with Psi easily developed brown color and fluorescence and was easily polymerized by cross-linking through the Maillard reaction. Furthermore, the Psi-modified OVA could develop a more excellent gelling property. It might be presumed that cross-linking chemistry and products subsequently formed through the Maillard reaction could contribute to the improved gelling properties of OVA by modification with Psi. These insights would have important implications for the creation of the desirable structure and physicochemical properties of sweetened food products using a rare sugar.

ABBREVIATIATIONS USED

OVA, ovalbumin; Ct, control sample; Glc, D-glucose; Fru, D-fructose; Psi, D-psicose; DTNB, 5,5′-dithiobis(nitrobenzoic acid); ANS, 8-anilino-1-naphthalenesulfonic acid; SDS-PAGE, sodium dodecyl sulfate gel electrophoresis; 2-ME, 2-mercaptoethanol; FT-IR, Fourier transform infrared; TNBS, trinitrobenzenesulfonic acid; CML, N^{ϵ}-carboxymethyllysine; AGEs, advanced glycation end products.

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