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Modulation of Physicochemical and Conformational Properties of Kidney Bean Vicilin (Phaseolin) by Glycation with Glucose: Implications for Structure−Function Relationships of Legume Vicilins

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ABSTRACT: The structure−function relationships of plant oligomeric globulins are still not fully recognized. The present work investigated the influence of glycation with glucose (at 1:50 and 1:100 protein/sugar molar ratios; incubation periods of 2.5, 5.0, and 10.0 h) on the physicochemical and conformational properties of kidney bean vicilin (phaseolin), with the aim of understanding the structure−function relationships of legume vicilins. Protein solubility (PS), surface charge (isoelectric point) and hydrophobicity (H_0), and secondary, tertiary, and/or quaternary conformations, as well as the emulsifying activities (emulsifying activity and emulsion stability indices, EAI and ESI) were evaluated. The 2.5 h incubation period of glycation led to least PS and highest H_0, and after that, the PS and H_0, on the contrary, gradually changed with increasing incubation period. The glycation increased the α-helix content and highly ordered secondary structures (α-helix + β-strand), as evidenced by far-UV circular dichroism (CD) spectroscopy. Combined analyses of differential scanning calorimetry, intrinsic emission fluorescence, and near-UV CD spectroscopy indicated that phaseolin underwent a tertiary conformation unfolding and subsequent rearrangement process (to form a new tertiary conformation), whereas the quaternary conformational flexibility progressively increased upon increasing degree of glycation. The conformation rearrangement was more distinct at the 1:100 molar ratio than at the 1:50 counterpart. The glycation at 5.0 and 10.0 h periods considerably increased the EAI, but only at the 1:50 molar ratio was the ESI progressively increased with the incubation period. These results confirmed that besides surface properties (e.g., PS and H_0), the flexibility in tertiary and/or quaternary conformations played a major role in the emulsifying properties of glycated vicilins. The findings would have important implications for understanding the structure−function relationships of legume oligomeric globulins, thus providing a direction to further improve the surface-related functional properties of these proteins.

KEYWORDS: phaseolin, 7S globulin, vicilin, glycation, conformational flexibility, emulsifying property, structure−function relationship

INTRODUCTION

The structure−function relationships of plant oligomeric proteins and legume 7S globulins in particular have been widely studied and generally recognized. For example, the role of N-linked glycans in the assembly and conformation, as well as some physicochemical properties, of vicilins (7S or 8S globulins) from soybean and red and mung beans, especially soy β-conglycinin, has been recognized.[1−5] However, knowledge about the structure−function relationships of these proteins is still very limited; for example, the importance of conformational characteristics for the functionalities is rarely addressed. The direct consequence of this limited knowledge is the inconsistency of the knowledge from different works performed by different researchers, even with the same protein materials and methods, as well as the difficulty to predict and modulate the functionalities of the proteins. One of the main factors causing this situation is that the conformations of plant oligomeric globulins are highly affected by the preparation process, which was often neglected in many previous works.

Kidney bean 7S globulins (phaseolin), obtained with acidic extraction at a high ionic strength (0.5 M NaCl), have been confirmed to keep their native conformations, to a large extent.[6] This fractionation technique can also be applied to obtain vicilins from other legume beans, for example, mung and red beans.[7,8] Using the acid-extracted vicilins (crude) as the starting materials, we have successfully purified and fractionated using an ion-exchange chromatographic technique different vicilins with different polypeptide constituents, from mung, red, and kidney beans. This provides us a strategy to investigate the physicochemical function−conformational feature relationships of the vicilins.[7−9] For example, we compared the physicochemical properties and the conformations of the vicilins with different polypeptide constituents from different legume varieties (kidney, red, and mung beans) and found good relationships between some selected physicochemical functions and the flexibility in their conformations; for example, the emulsifying capacity and emulsion stability of these vicilins are closely related to the flexibility in their tertiary and quaternary conformations, respectively.[9]

In contrast with the vicilins from mung and red beans, kidney bean phaseolin is much more homogeneous in polypeptide

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constituents and is thermally stable, with much higher flexibility in quaternary conformation but lower flexibility in tertiary conformation.[8] In a previous work comparing the physicochemical properties of 7S and 11S globulins from various legume sources, obtained with extraction at pH 8.0 and selective ammonium sulfate precipitation, it has also been found that the 7S globulin of French bean (kidney bean) exhibited excellent solubility at pH 4.2−7.0 and a low ionic strength (μ = 0.08) and excellent emulsion stability at pH 7.6 and μ = 0.08.[10] This seems to be consistent with the peculiar structural characteristics of this 7S globulin.[11]

Another strategy to investigate the structure−function relationships of vicilins is to characterize the changes in their physicochemical and conformational properties, through the modifications of the active groups on the surface of the proteins. The glycation with mono- or bisaccharides has been proved to be an effective technique to modify the surface properties and conformations of food proteins, through a reductive alkylation of lysine ε-amino groups.[12] However, to date, only a few papers in the literature are available addressing the modifications of functional and structural properties of several oligomeric globulins, for example, pea 11S and 7S globulins,[13−16] and soy 11S glycinin.[17] In these works, little information was given about their structure−function relationships.

Thus, this study aimed (1) to characterize the influence of glycation with glucose at two protein/sugar molar ratios (1:50 and 1:100) for several incubation periods (2.5−10.0 h) on the physicochemical and conformational properties of kidney bean phaseolin (7S vicilin) and (2) to establish the structure−function relationships of vicilins. The surface hydrophilicity and/or hydrophobicity and secondary, tertiary, and quaternary conformations as well as the emulsifying activities were evaluated.

MATERIALS AND METHODS

Materials. Red kidney bean, cultivated in Shandong province or the northeastern area of China, was purchased from a local supermarket (Guangzhou, China). The seeds were soaked in deionized water for 12 h at 4 °C and dehulled manually. The dehulled seeds were freeze-dried, ground, and defatted by Soxhlet extraction with hexane to produce the defatted flour. 1,8-Anilinonaphthalenesulfonate (ANS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Fitzgerald Industries International Inc. (Concord, MA). Low molecular weight protein markers and β-mercaptoethanol (2-ME) were purchased from Shanghai DINGUO Biotech. Co., Ltd. (China). All other chemicals used were of analytical grade.

Preparation of Purified Phaseolin. The crude phaseolin sample was prepared according to the process described by Hall and others,[18] with slight modifications. The defatted flour (5.0%, w/v) was dispersed in a 0.5 M NaC1 solution containing 0.025 M HCl (pH 3.5). The resultant dispersion was gently stirred at 25 °C for 2 h. The slurry was centrifuged (10000*g*, 20 min) at 4 °C in a CR22G centrifuge (Hitachi Co., Japan) and the supernatant diluted with 5-fold volumes of deionized water (0−4 °C). Then, the obtained precipitate was collected by centrifugation at 12000*g* for 20 min at 4 °C. The pellet was dissolved in 0.5 M NaCl solution and reprecipitated twice as above. The last obtained precipitate was finally dissolved in 0.5 M NaCl solution, dialyzed against deionized water at 0−4 °C for 48 h, and then lyophilized to produce the crude phaseolin.

The crude phaseolin was further fractionated using DEAE-Sepharose fast flow column chromatography with an AKTA Purifier (GE Co. Ltd., USA), as described in our previous work.[8] Elution was performed using 50 mM phosphate buffer (pH 7.0) with a gradient of 0−0.5 M NaCl, at a flow rate of 2.5 mL/min. The eluents were collected at 10 mL per tube. The collected fractions, pooled according to the requirement, were resolved by sodium dodycyl sulfate−polyacryamide gel electrophoresis (SDS-PAGE). The major fractions were collected and combined and then dialyzed against deionized water at 4 °C and further freeze-dried to produce the purified phaseolin. The protein content (N × 5.85) of the purified phaseolin was about 93% (wet basis) as determined according to the micro-Kjeldahl method.

Glycation Reaction and Preparation of Glycated Phaseolin Samples. Phaseolin and glucose were fully solubilized in 10 mM phosphate buffer (pH 7.0) at protein/glucose molar ratios of 1:100 and 1:50, respectively, and then freeze-dried. The freeze-dried samples were then equilibrated in saturated KBr solution (relative humidity (RH) 79%) at room temperature for 1 day. The equilibrated samples at the same RH were further stored at 60 °C for 2.5, 5.0, and 10.0 h, respectively. After that, the samples were removed, dispersed in deionized water, and dialyzed against deionized water at 4 °C for 48 h. Finally, the dialyzed samples were freeze-dried and stored at −20 °C prior to further analyses.

Determination of Extent of Glycation. The extent of glycation of glycated phaseolin samples was indirectly determined through the changes in free amino groups (mainly ε-amino groups), as analyzed using the TNBS method of Habeeb.[19] In brief, protein dispersions (1.0%) were prepared in 50 mM phosphate buffer (pH 8.5) containing 50 mM NaCl. One milliliter of a 0.1% (w/v) TNBS solution was mixed with 1 mL of protein dispersion, and the resultant mixtures were incubated in a 60 °C water bath for 2 h and then cooled to room temperature. One milliliter of 10% SDS and 0.5 mL of 1.0 M HCl were added to the protein mixtures. Then, the absorbance of the final mixtures was read at 335 nm in a spectrophotometer against a reagent blank. The absorbance of native phaseolin was defined as 100% free amino groups, and the degree of glycation was calculated by percent decrease in absorbance relative to that of native phaseolin.

SDS-PAGE. SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli[20] using a 12% separating gel and a 4% stacking gel. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% trichloroacetic acid and destained in a methanol−water solution containing 7% (v/v) acetic acid and 40% (v/v) methanol. The test protein samples for reducing SDS-PAGE experiments were prepared by dissolving the freeze-dried samples in the electrophoretic sample buffer (1×), namely, 0.25 M Tris-HCl buffer (pH 8.0) containing 2.0% (w/v) SDS, 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol, and 10% (v/v) 2-ME.

Isoelectric Point (p*I*) Determination. The p*I* of various phaseolin samples was determined on the basis of the zeta potential profiles using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, U.K.) in combination with a multipurpose autotitrator (model MPT-2, Malvern Instruments Ltd.). Freshly prepared protein dispersions were diluted to 2 mg/mL with deionized water and filtered through a 0.45 μm HA Millipore membrane prior to analysis. Titration was performed from pH 10.0 to 2.0 with 0.25 and/or 0.025 M NaOH or HCl under constant stirring.

Determination of Surface Hydrophobicity (H_0). The H_0 was determined with the fluorescence probe ANS^- according to the method of Haskard and Li-Chan.[21] Serial dilutions in 10 mM phosphate buffer (pH 7.0) were prepared with the protein samples (stock solutions; 1.5%, w/v) to a final concentration of 0.004−0.02% (w/v). ANS^- solution (8.0 mM) was also prepared in the same buffer. Twenty microliters of ANS^- solution was added to 4 mL of each dilution, and the fluorescence intensity (FI) of the mixture was measured at 390 nm (excitation) and 470 nm (emission) using an F4500 fluorescence spectrophotometer (Hitachi Co., Japan). The initial slope of the FI versus protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of H_0.

Protein Solubility (PS) Profiles as a Function of pH. An aqueous solution (1.0%, w/v) of protein samples was stirred magnetically for 30 min, and then, with either 0.5 M HCl or 0.5 M NaOH, the pH of the solutions was adjusted to the desired values. After 30 min of stirring, the pH was readjusted if necessary. Then the sample was centrifuged at 8000*g* for 20 min at 20 °C in a CR22G centrifuge (Hitachi Co., Japan). After appropriate dilution, the protein content of the supernatant was determined according to the Lowry method using BSA as the standard.[22] The PS was expressed as grams of soluble protein per 100 g of total protein. All determinations were conducted three times.

Determination of Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI). EAI was determined according to the method of Pearce and Kinsella,[23] as modified by Cameron and others.[24] For emulsion formation, 15 mL of 0.05−1.0% (w/v) protein solutions in 50 mM phosphate buffer (pH 7.0) and 5 mL of corn oil were homogenized in an Ultra-Turrax T25 digital homogenizer (IKA Co., Germany) at 24000 turns/min for 1 min. Fifty microliters of emulsion was taken from the bottom of the homogenized emulsion, immediately after homogenization, and diluted (1:100, v/v) in 0.1% (w/v) SDS solution. After 5 s of shaking in a vortex mixer, the absorbance of dilute emulsions was read at 500 nm using a Spectrumlab 22PC spectrophotometer (Shanghai Lengguang Technology Co. Ltd., Shanghai, China). EAI and ESI values were calculated using eqs 1 and 2

$$\text{EAI}\ (\text{m}^2/\text{g}) = \frac{2 \times 2.303 \times A_0 \times \text{DF}}{c \times \phi \times (1-\theta) \times 10000} \quad (1)$$

$$\text{ESI}\ (\text{min}) = \frac{A_0}{A_0 - A_{10}} \times 10 \quad (2)$$

where DF is the dilution factor (100), c is the initial concentration of protein (g/mL), ϕ is the optical path (0.01 m), θ is the fraction of oil used to form the emulsion (0.25), and A_0 and A_{10} are the absorbances of diluted emulsions at 0 and 10 min, respectively. Measurements were performed in at least quadruplicate.

Differential Scanning Calorimetry (DSC). The thermal transition of various protein samples was examined using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE). Approximately 2.0 mg of the samples was weighed into aluminum liquid pans (Dupont), and 10 μL of 50 mM phosphate buffer (pH 7.0) was added. The pans were hermetically sealed and heated from 20 to 110 °C at a rate of 5 °C/min. A sealed empty pan was used as a reference. Onset temperature (T_o), peak transition or denaturation temperature (T_d), enthalpy change of the endotherm (ΔH), and cooperativity, represented by the width at half peak height ($\Delta T_{1/2}$), were computed from the thermograms by Universal Analysis 2000, version 4.1D (TA Instruments-Waters LLC). All experiments were conducted in triplicate. The sealed pans containing protein samples and buffers were equilibrated at 25 °C for >6 h.

Far-UV and Near-UV Circular Dichroism (CD) Spectroscopy. Far-UV and near-UV CD spectra were obtained using an MOS-450 spectropolarimeter (BioLogic Science Instrument, France). The far-UV CD spectroscopic measurements were performed in a quartz cuvette of 2 mm with a protein concentration around 0.1 mg/mL in 10 mM phosphate buffer (pH 7.0) or purified water at pH 3.0 and 9.0. The sample was scanned from 190 to 250 nm. The near-UV CD spectroscopy measurements were performed in a 1 cm quartz cuvette with a protein concentration around 1.0 mg/mL. The sample was scanned over a wavelength range from 250 to 320 nm. For both measurements, the spectra were an average of eight scans. The following parameters were used: step resolution, 1 nm; acquisition duration, 1 s; bandwidth, 0.5 nm; sensitivity, 100 millidegrees. The cell was thermostated with a Peltier element at 25 °C, unless specified otherwise. The concentration of the proteins was determined according to the Lowry method[22] using BSA as the standard. Recorded spectra were corrected by subtraction of the spectrum of a protein-free buffer. A mean value of 112 for the amino acid residue was assumed in all calculations, and CD measurements were expressed as mean residue ellipticity (θ) in degrees·cm^{-2}·dmol^{-1}. The secondary structure compositions of the samples were estimated from the far-UV CD spectra using the CONTIN/LL program in CDPro software, using 43 kinds of soluble proteins as the reference set.[25] Data are the means of duplicate measurements.

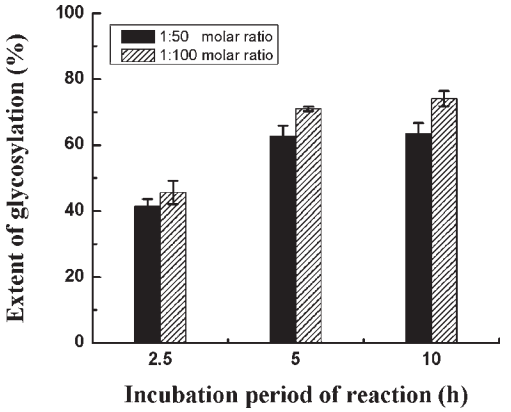

Figure 1. Extent of modification of glycated phaseolin samples, obtained with glucose at 1:50 and 1:100 protein/sugar molar ratios for various incubation periods (2.5, 5.0, and 10.0 h). Each bar is the mean and standard deviation of duplicate measurements.

Intrinsic Emission Fluorescence Spectroscopy. Intrinsic emission fluorescence spectra of various phaseolin samples at various pH values were determined with the F4500 fluorescence spectrophotometer (Hitachi Co., Japan). Protein dispersions (0.15 mg/mL) were prepared in 10 mM phosphate buffer (pH 7.0). Protein solutions were excited at 295 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 5 nm for both excitation and emission.

Statistical Analysis. An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Degree of Glycation. Glycation was carried out with glucose, at two protein/sugar molar ratios (1:50 and 1:100), for three periods of incubation time (2.5, 5.0, and 10.0 h). As expected, the degree of glycation increased more rapdily during the initial incubation of reaction (e.g., 0−2.5 h), and the rate of increase became slower upon further incubation (e.g., 2.5−5.0 h) and even reached a "saturation" (up to 10 h) (Figure 1). This reflects that the glycation reaction during the initial period of phaseolin might occur on the ε-amino groups exposed on the surface, and when amounts of sugar moieties are attached, the structure of the protein will unfold and, subsequently, some new reactive groups are exposed to be accessible for further glycation reaction. Similar phenomena of the dependence of degree of glycation upon the incubation time have been observed for glycated pea legumin (11S globulin),[13] soy 11S glycinin,[17] and soy protein isolate.[26] The degree of glycation at comparable molar ratios (e.g., 1:50−1:44) of glycated phaseolin was much higher than that of glycated soy glycinin.[17] This partially confirms that the glycation reaction occurs much more easily at higher temperatures, because in the present work, a temperature of 60 °C was applied, whereas in the Achouri et al. work 50 °C was applied.

At both molar ratios (1:50 and 1:100), an incubation time of 5.0 h was enough to obtain the maximal degree of glycation

(Figure 1). The degrees of glycation (about 40%) at both molar ratios were similar after incubation of 2.5 h, whereas at longer incubation periods, the degree of glycation at the 1:100 molar ratio was slightly but significantly higher than that at 1:50 molar ratio (Figure 1).

Physiochemical Characteristics. *Molecular Weight of the Polypeptides.* Figure 2 shows reducing SDS-PAGE profiles of native and glycated phaseolin samples. As expected, the native phaseolin was mainly composed of two polypeptides with molecular weights of about 50 (major) and 27 (minor) kDa, respectively (Figure 2, lane 1). This agrees well with previous literature about the electrophoretic mobility of polypeptides of phaseolin.[8,27] We can see that the glycation treatment resulted in decreased electrophoretic mobility of both polypeptides, and the decrease in mobility was greater in magnitude at higher extents of glycation (Figure 2, lanes 2−7). For example, the mobility of the polypeptides was slowest for the phaseolin glycated at a 1:100 molar ratio for 5.0−10.0 h, for which the degree of glycation was highest (Figure 2, lanes 6 and 7). Similar phenomena have been observed for glycated pea legumin,[13] for which the decrease in electrophoretic mobility was attributed to an increase in hydrodynamic volume of the molecules. This can be also indirectly corroborated by a previous observation that the glycation did not change the electrophoretic mobility of pea vicilin (7S globulins),[16] because in this work, the degree of glycation was much lower (4.7−13.4%) relative to that (>40%) in the present work.

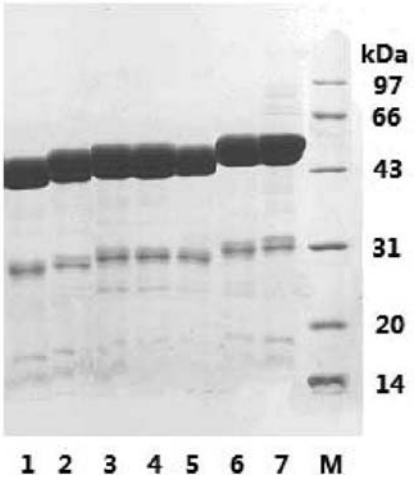

Figure 2. Reducing SDS-PAGE profile of native and glycated phaseolin samples. Lanes: 1, native phaseolin; 2−4, glycated samples at 1:50 molar ratio for 2.5, 5.0, and 10.0 h, respectively; 5−7, glycated samples at 1:100 molar ratio for 2.5, 5.0, and 10.0 h, respectively; M, protein markers.

Isoelectric Point (pI) and Surface Hydrophobicity (H_0). The glycation, mainly through the attachment of sugar residues to ε-amino groups of the lysyl residues in proteins, usually causes the increases in net negative charge and hydroxyl groups on the surface of the proteins. Thus, the surface hydrophilicity/hydrophobicity balance will be changed upon glycation, which greatly affects the surface properties of phaseolin. The isoelectric point (p*I*) and surface hydrophobicity (H_0) are two important parameters reflecting the charge and surface hydrophobic properties. Table 1 summarizes the p*I* and H_0 data of native and glycated phaseolin samples. We can see that the glycation treatment with incubation periods to 10.0 h resulted in a slight but progressive decrease in p*I* from 4.85 (control) to 4.27 and 4.45 for 1:100 and 1:50 molar ratios, respectively (Table 1). The p*I* shift of proteins by the glycation (toward lower values) has also been observed in many previous works,[14,15,17,30] reflecting the decrease in positive charge density of the proteins by derivatization of lysyl residues. The higher extent of decrease in p*I* at the 1:100 molar ratio seems to be consistent with a higher degree of glycation (Figure 1), indicating more blockage of ε-aminolysyl groups.

In contrast, the glycation treatment led to increases in H_0, with the extent of the increase depending on the applied protein/sugar molar ratio and incubation period of time (Table 1), indicating increased hydrophobic clusters on the surface of the proteins. The 2.5 h incubation period resulted in the highest H_0, and with increasing incubation period (5.0−10.0 h), the H_0 on the contrary decreased (Table 1). At incubation periods of 5.0 or 10.0 h, the H_0 at 1:50 molar ratio was distinctly higher than that at the 1:100 counterpart. The change pattern in H_0 upon increasing degree of glycation, observed in the present work, is slightly different from that observed for rice proteins glycated with glucose (wet heating)[28] and soy 11S glycinin,[17] for which, in general, the H_0 gradually decreased. This difference is clearly due to the difference in nature of the applied protein. In the present work, the applied protein (phaseolin) was much less denatured (with a H_0 15 times less than that of rice protein[28]).

Table 1. Isoelectric Point (p*I*), Surface Hydrophobicity (H_0), and Some DSC Characteristics of Native and Glycated Phaseolin Samples[a]

protein samples				DSC characteristics[b]			
molar ratio	incubation period (h)	p*I*	H_0	T_m (°C)	T_d (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)
native		4.85	167.9	85.5 ± 0.5 d	91.5 ± 0.3 d	13.6 ± 0.3 a	5.7 ± 0.1 c
1:50	2.5	4.78	332	90.0 ± 0.2 c	97.0 ± 0.1 c	13.7 ± 0.1 a	6.6 ± 0.5 b
	5.0	4.56	236.7	90.4 ± 0.3 c	97.3 ± 0.2 c	13.0 ± 0.2 b	5.7 ± 0.7 c
	10.0	4.45	248.3	90.8 ± 0.2 c	97.0 ± 0.1 c	14.1 ± 0.3 a	5.6 ± 0.1 c
1:100	2.5	4.62	305	91.1 ± 0.0 b	97.7 ± 0.2 c	13.1 ± 0.0 b	7.9 ± 0.2 a
	5.0	4.38	197.6	92.6 ± 1.2 a	99.4 ± 0.3 a	12.2 ± 0.4 c	6.2 ± 0.3 b
	10.0	4.27	196	91.6 ± 0.1 ab	98.8 ± 0.0 b	11.9 ± 1.0 c	6.1 ± 0.1 b

[a] Data are the means and standard deviations of duplicate measurements. Different letters (a−d) indicate significant difference at the $p < 0.05$ level among various samples. [b] T_m, onset temperature of denaturation of the endotherm; T_d, denaturation peak temperature; ΔH, enthalpy change of the main endotherm; $\Delta T_{1/2}$, width at half peak height of the endotherm.

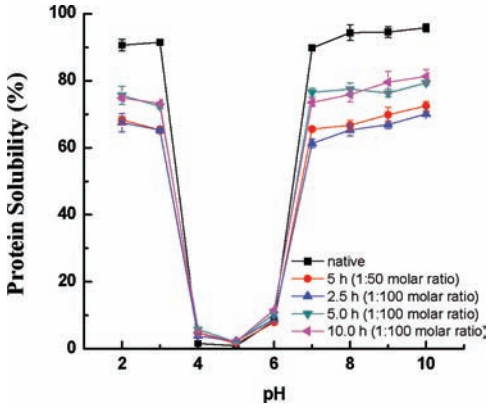

Figure 3. Protein solubility profiles of native and glycated phaseolin samples as a function of pH. Each data point is the mean and standard deviation of duplicate measurements.

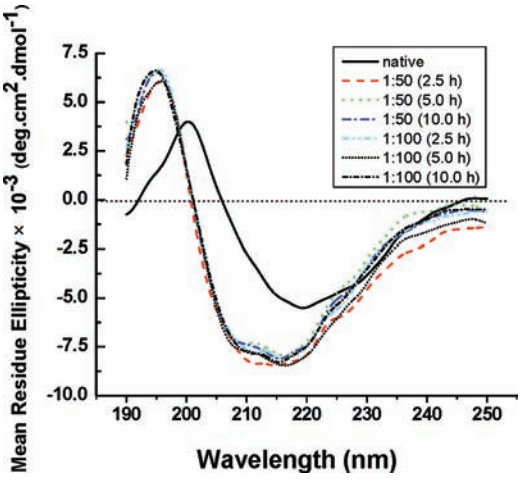

Figure 4. Typical far-UV CD spectra of native and glycated phaseolin samples at pH 7.0. Glycated samples were prepared at 1:50 and 1:100 protein/sugar molar ratios for 2.5, 5.0, and 10.0 h, respectively.

Protein Solubility (PS) as a Function of pH. The changes in surface hydrophilicity/hydrophobicity balance of phaseolin by glycation can be reflected in changes in its solubility, because the solubility of a protein can be understood as the manifestation of the equilibrium between the protein−solvent (hydrophilic) and the protein−protein (hydrophobic) interaction.[29] All of the test samples exhibited minimal PS values at around pH 4.0−5.0 (which is consistent with the p*I* data; Table 1) and a progressively increased PS at pH deviating from this range, for example, >6.0 or <4.0 (Figure 3). At pH ≥7.0 or at pH ≤3.0, the PS of native phaseolin was >90%, indicating that the intermolecular electrostatic repulsion interactions played a vital role in its PS at pH deviating from the p*I*.

Figure 3 illustrates that the glycation treatment resulted in considerable decreases in PS of phaseolin at pH deviating from the p*I*. The observation seems to be in contrast with the general viewpoint that the attachment of hydrophilic sugar residues to positively charged groups would be favorable for the improvement of the solubility of the protein.[12] In fact, this phenomenon well confirms that besides the electrostatic repulsion (hydrophilic nature), the surface hydrophobic nature is also an important parameter for the solubility of a protein. From this view of point, the decreases in PS by glycation can be largely attributed to enhanced attractive protein−protein interactions, especially hydrophobic interactions, as evidenced by increased H_0 for glycated phaseolin samples (Table 1).

The glycation-induced decreases in PS, at pH deviating from the p*I*, were closely dependent on incubation period (or extent of glycation) and protein/sugar molar ratio (Figure 3). At a 1:100 molar ratio, for example, maximal decrease in PS (about 32%; relative to native counterpart) was observed for glycated (2.5 h) phaseolin; the glycation with increasing incubation period up to 5.0 or 10.0 h, on the contrary, increased the PS, for example, at pH 7.0, as compared to that with a 2.5 h incubation period (Figure 3). The change pattern in PS of phaseolin by the glycation is contrary to that of the H_0 data (Table 1), further suggesting that the solubility of glycated phaseolin samples mainly depended on their intermolecular hydrophobic interactions. The opposite relationship between the PS and H_0 can be also observed for the phaseolin samples glycated at different molar ratios, for example, the PS at pH ≥7.0 was higher at the 1:100 molar ratio than that at the 1:50 counterparts, whereas the opposite was true for H_0 at comparable incubation periods (Figure 3 and Table 1).

Contrasting results have been reported for glycated pea 7S globulins (vicilins) and 11S legumin,[14,15] soy 11S glycinin,[17] and rice proteins,[28] for which it was observed that the glycation resulted in increased PS and/or decreased H_0. One of the possible factors causing the differences between these previous works and the present work is that the modification of solubility by glycation is highly dependent on the nature of the applied protein. If the solubility of an unmodified protein (e.g., phaseolin in the present work) is good itself, the glycation will be unfavorable for the solubility, because the attachment of sugar moieties may induce structural unfolding, and as a consequence, the hydrophobic clusters will be exposed and the aggregation favored. Whereas in the case of the proteins with poor solubility, for example, the PS of rice proteins being only about 10% even at pH 11,[28] the attachment of hydrophilic groups will greatly increase the hydrophilic nature of the proteins, thus increasing the solubility. This argument is further confirmed by the fact that many previous works addressing the improvement of solubility by glycation, reviewed by Oliver et al.,[12] were conducted with proteins having poor solubility, for example, fish, scallop, and carp myofibrillar proteins and myosin.

Conformational Characteristics. *Secondary Conformation.* To confirm the conformational changes of phaseolin, caused by the glycation, the conformations at secondary, tertiary, and/or quaternary levels of native and glycated phaseolin samples were characterized and even compared. The secondary conformation, especially α-helices and β-sheets of the proteins, can be well characterized using far-UV CD spectroscopy in the wavelength range from 190 to 260 nm.[30] Figure 4 shows typical far-UV CD spectra of native and glycated phaseolin samples at pH 7.0. The native phaseolin exhibited a far-UV CD spectrum with a prominent negative band centered at around 218 and a relatively minor positive band at about 200 nm (Figure 4). These features are sufficient indicators of a highly ordered structure, most probably of the β types.[31,32] The secondary structure composition of native phaseolin was approximately as follows: α-helix, 7.5%; β-strand, 36.3%; β-turn, 22.3%; and random coil, 33.9% (Table 2), as calculated according to the CONTIN/LL program in CDPro software.[25]

The glycation resulted in a marked shift of both the negative and positive bands toward lower wavelength, and the ellipicity of

Table 2. Secondary Structure Compositions and Intrinsic Emission Spectrum Characteristics of Native and Glycated Phaseolin Samples at pH 7.0[a]

protein samples		secondary structure composition (%)				characteristics of fluorescence spectra	
molar ratio	incubation period (h)	α-helix[b]	β-strand[c]	turns	random coil	λ_m[d] (nm)	maximum fluorescence intensity
native		7.5	36.3	22.3	33.9	331.2	3092
1:50	2.5	9.1	35.4	23.0	32.5	332.4	3202
	5.0	10.3	34.1	22.4	33.2	333.6	3109
	10.0	9.7	34.6	22.3	33.4	334.2	3010
1:100	2.5	9.5	35.1	23.5	31.9	332.8	3301
	5.0	11.0	35.3	22.0	31.7	333.6	3223
	10.0	11.4	35.9	22.1	30.6	334.5	3110

[a] The secondary structure composition was calculated from far-UV CD spectra using the CONTIN/LL program in CDPro software. [b] Combined regular and distorted α-helix. [c] Combined regular and distorted β-strands. [d] Wavelength at maximum fluorescence intensity.

these bands considerably increased, but there were no remarkable differences among various glycated samples (Figure 4), indicating that the secondary conformation was distinctly changed by the treatment. Basically, the glycation increased the α-helix content and highly ordered structure content (α-helix + β-strand), mainly at the expense of β-strand and/or random coil contents (Table 2). The changes in secondary structure composition were more distinct at the 1:100 molar ratio than at the 1:50 counterpart. For example, the highly ordered structure content was on average increased by 6.4 and 2.5% after glycation at 1:100 and 1:50 molar ratios, respectively.

Similar secondary structure changes by the glycation have been observed for pea 11S legumin, for which the secondary structure was evaluated by the same CD technique, but with a different fitting method.[13] However, in another previous work, no distinct changes in secondary structure composition were observed for pea 7S globulin (vicilin), glycated at relatively low degrees of modification (7.5−13.4%[16]). Thus, it can be reasonably suggested that distinct secondary conformational changes might occur only at a high degree of glycation, for example, >40%.

Tertiary and/or Quaternary Conformation. 1. DSC. The thermal transition as detected by DSC reflects the state and nature of conformations (mainly tertiary conformation) in the proteins, mostly related to disruption of hydrogen bonds. All of the test phaseolin samples exhibited only a prominent endothermic peak in the DSC profiles, clearly attributed to the thermal denaturation of phaseolin subunits (data not shown). The peak transition temperature (T_d) of native phaseolin at pH 7.0 was about 91.5 °C (Table 1), which is approximately comparable to that of crystalline phaseolin.[33]

The T_d, as well as the onset temperature of denaturation (T_o), was considerably increased (e.g., to 97.0−99.4 °C for T_d) by the glycation (Table 1), indicating a remarkable increase in thermal stability, or tertiary conformational stability. This is consistent with the general observation that the glycation or addition of N-linked glycans may increase the surface hydrophilic nature and net charge of the proteins, thus favoring the stability in their tertiary conformation.[5,16] The extent of the increases in T_d was also dependent on the applied molar ratio and the incubation period (Table 1). For example, at the 1:50 molar ratio, glycation with different incubation periods led to similar increases in T_d (to 97.0−97.3 °C), whereas at the 1:100 molar ratio, the T_d was even further increased to 99.4 °C after glycation with a 5.0 h incubation period (Table 1). The data indicated that the tertiary conformation stability of phaseolin was to a higher extent increased at 1:100 molar ratio than at the 1:50 counterpart.

On the other hand, the ΔH was almost unaffected by glycation at the 1:50 molar ratio, even with a 10.0 h incubation period, whereas at the 1:100 molar ratio, it was slightly but progressively decreased by the treatment, as the incubation period increased from 0 to 10.0 h (Table 1). The ΔH usually represents the proportion of undenatured protein in a sample or content of ordered structure.[34] Thus, the data clearly indicated that glycation at the 1:100 molar ratio led to a much higher extent of structural changes than at the 1:50 counterpart. The width at half peak height of the endotherm ($\Delta T_{1/2}$) was wholly to a slight extent increased by the treatment (Table 1), indicating a slight change in cooperativity of transition from native to denatured state.[35]

2. Intrinsic Emission Fluorescence Spectroscopy. The intrinsic (or Trp) fluorescence spectrum is determined chiefly by the polarity of the environment of the Trp residues and provides a sensitive means of monitoring conformational changes in proteins and protein−protein as well as ligand−protein interactions.[37] It is generally recognized that the fluorescence emission maximum (λ_m) suffers a red shift when the Trp chromophores become more exposed to solvent, and the quantum yield of fluorescence decreases when the chromophores interact with quenching agents either in a solvent or in the protein itself. The Trp fluorescence spectrum of native phaseolin exhibited a λ_m of 331.2 nm (Figure 5A and Table 2), which is a typical fluorescence profile of Trp residues located in a hydrophobic environment, such as the interior of the globulin.[38] The maximal fluorescence (3092) is approximately 18 times that (175) of kidney protein isolate,[39] reflecting a considerable difference in the conformational characteristics of the proteins obtained with different processes.

The glycation resulted in a progressive red shift (up to 3.0−3.5 nm) in λ_m as the incubation period increased from 2.5 to 10 h (Table 2). The magnitudes in red shift at comparable incubation periods were similar at 1:50 and 1:100 molar ratios. The data suggest that the Trp chromophores became exposed to a more hydrophilic microenvironment when the extent of glycation was increased. In contrast, the magnitude of maximal fluorescence was slightly affected (Figure 5A and Table 2). However, it can be still observed that glycation with a 2.5 h incubation period resulted in a maximal increase in the fluorescence

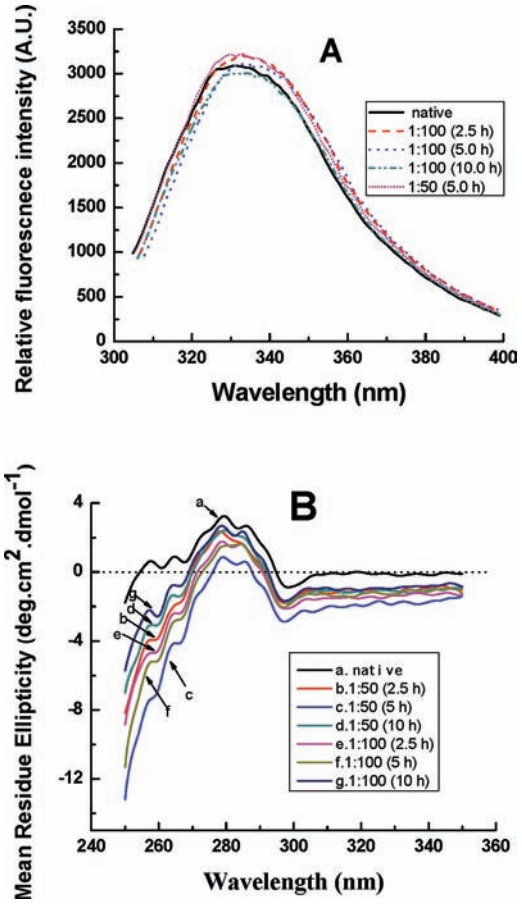

Figure 5. Intrinsic emission fluorescence (A) and near-UV CD (B) spectra of native and glycated phaseolin samples at pH 7.0. The glycated samples were prepared at 1:50 and 1:100 molar ratios, after various incubation periods (2.5−10 h).

intensity, and upon extended incubation periods, the magnitude in maximal fluorescence on the contrary gradually decreased. The maximal fluorescence data seem to be in contrast to the λ_m data (Table 2), as it is generally recognized that a red-shift phenomenon is associated with a decrease in maximal fluorescence.[36] A reasonable explanation for this "paradox" may be that the attachment of sugar moieties to the protein increases the polar nature of microenvironment of Trp hydrophobic chromophores, but it renders the Trp hydrophobic chromophores more buried within the molecules, thus resulting in a lower extent of interaction with quenching agents. The increased tertiary conformation stability has been evidenced by the DSC data (Table 1).

3. Near-UV CD Spectroscopy. The changes in tertiary and/quaternary conformations of phaseolin by glycation were further evaluated using near-UV CD spectroscopy. The actual shape and magnitude of the near-UV CD spectrum of a protein will depend on the number of each type of aromatic amino acid present (Phe, Tyr, and Trp), their mobility, and the nature of their environment (H-bonding, polar groups, and polarizability), as well as their spatial disposition in the protein.[39] The nature of the environment of hydrophobic chromophores in an oligomeric globulin (with a quaternary structure) not only depends on the flexibility of tertiary conformation (polypeptide or subunit) but also, more importantly, is related to the intersubunit interactions that determine the flexibility of quaternary conformation. This has been confirmed in our previous work, in which the difference in near-UV ellipicity magnitude of the hydrophobic chromophores for various vicilins has been largely attributed to the difference in the flexibility in their quaternary conformation.[9] The flexibility in quaternary conformation of native phaseolin is much higher than that of other viclins (from red and mung beans), as evidenced by the much reduced magnitude of its near-UV ellipicity.[9]

The low magnitude of near-UV ellipicity (in the wavelength range of 250−300 nm) of the hydrophobic chromophores for native phaseolin is similarly observed in the present work (Figure 5B). Interestingly, the near-UV ellipicity of the hydrophobic chromophores was further decreased by glycation, with the extent of the decreases depending on the applied molar ratio and incubation period (Figure 5B). For example, the ellipicity of the hydrophobic chromophores progressively decreased as the incubation period increased to 5.0 h, at any applied protein/sugar molar ratio. The decreases in the ellipicity indicated increased hydrophilicity of the hydrophobic chromophores, possibly due to the glycation-induced conformational changes at tertiary and/or quaternary levels. We should keep in mind that the Trp fluorescence analyses had indicated that glycation led to increased tertiary conformation stability (as evidenced by the red shift in λ_m and increased maximum fluorescence intensity; Table 2). As usual, the increase in tertiary conformation stability is associated with increased magnitude of near-UV ellipicity of the proteins, because more hydrophobic groups will shift to a more hydrophobic microenvironment. However, the actual situation is the reverse. Thus, the decreases in near-UV ellipicity can be more specifically attributed to increased flexibility in the quaternary conformation of the phaseolin.

On the other hand, the influence of increased tertiary conformation stability on the magnitude of near-UV ellipicity can be still observed, at longer incubation periods of 5.0−10.0 h. For example, we can see that increasing the incubation period from 5.0 to 10.0 h, on the contrary, resulted in increased ellipicity of the hydrophobic chromophores, but the ellipicity at 10.0 h of incubation was still lower than that of the control (native sample) (Figure 5B). This phenomenon is consistent with the DSC and Trp fluorescence data (Table 2 and Figure 5A), indicating increased stability of the tertiary conformation by extensive glycation (10.0 h; as compared to the 5.0 h incubation period).

Proposed Mechanism for Glycation-Induced Changes in Conformations. Together with the H_0 and PS data (Table 1 and Figure 3), all of the conformational analyses suggest that with increasing degrees of glycation, the phaseolin might undergo a tertiary structural unfolding process, followed by a rearrangement of unfolded structures (and even formation of a new tertiary conformation). During the 2.5 h incubation period the glycation reaction seems to largely occur on ε-amino groups on the surface of the protein (as evidenced by higher increases in the extent of glycation after this period; Figure 1). As a direct result of decreased ε-amino groups and attachment of hydrophilic sugar moieties, the relative content of random coil (usually accessible to the solvent) distinctly decreases, and concomitantly, the more ordered structure (α-helix + β-strand) content increases (evidenced by far-UV CD data; Table 1 and Figure 4). Accordingly, the tertiary conformation also markedly changes and even becomes unfolded upon glycation, and finally, more hydrophobic clusters initially buried within the molecules are exposed. The evidence includes the highest H_0, lowest PS at pH deviating from the pI, remarkable increases in T_d (relative to native phaseolin),

and distinct decreases in near-UV CD ellipicity at 2.5 h of incubation (Figures 3 and 5 and Tables 1 and 2).

Upon further increase of the incubation period (e.g., up to 5.0 h), most of the exposed ε-amino groups have been glycated, and even some of the initially buried ε-amino groups become accessible to the sugars, as a result of tertiary structural unfolding and are further glycated. In this regard, a higher sugar level seems to be more favorable for the "additional" glycation (of initially buried ε-amino groups) (Figure 1). The attachment of more hydrophilic sugar moieties further changes the surface hydrophilicity/hydrophobicity balance, which seems to favor the structural rearrangement of unfolded conformation, and even forms a new and stable tertiary conformation. Evidence for the tertiary conformational rearrangement includes distinct decreases in H_0, increased PS at pH deviating from the p*I*, and a red shift but no distinctly changed intensity in fluorescence (as the incubation period is increased from 2.5 to 5.0 h; Figure 3 and Tables 1 and 2). The newly formed tertiary conformation becomes more compacted and ordered upon further increase of the incubation period (10.0 h). The increase in near-UV CD ellipicity at 10.0 h (relative to 5.0 h) (Figure 5B) well supports this argument.

The rearrangement of tertiary conformation is more favorable for the glycated phaseolin samples at the 1:100 molar ratio than at the 1:50 counterpart, as evidenced by further distinct increases in T_d after 5.0 or 10.0 h, as well as a relatively lower extent of the change in near-UV CD ellipicity at the 1:100 molar ratio (Table 1 and Figure 5B). The PS and H_0 data, for example, higher PS (at pH 7.0 or above) and lower H_0 values at the 1:100 molar ratio than at the 1:50 counterpart (Table 1 and Figure 3), also support this argument.

In contrast with the decreased flexibility in tertiary conformation, the flexibility of quaternary conformation progressively increases with increasing degree of glycation. This is evidenced by a progressive decrease in near-UV CD ellipicity with increasing incubation period from 0 to 5.0 h (Figure 5B). The increase in the ellipicity at 10.0 h (relative to 5.0 h) reflects that the tertiary conformational change plays a more important role than quaternary conformational change.

Emulsifying Functionality and Its Structure−Function Relationships. *Emulsifying Activities (EAI and ESI).* The functionalities in general and emulsifying properties in particular of legume storage proteins are essentially determined by their physicochemical and conformational properties, including molar mass, hydrophobicity/hydrophilicity balance, and solubility, as well as conformational stability.[40] Figure 6 shows the EAI and ESI values of native and glycated phaseolin samples at pH 7.0. The EAI usually represents the ability of the protein to help the dispersion of the oil phase into the water phase.[23] The EAI value of native phaseolin is about 190 m^2/g (Figure 6A), much higher than that reported for other native vicilins from pea and mung and red beans,[9,15] suggesting that this vicilin exhibited an excellent ability to form an emulsion. We can see that, at any protein/sugar molar ratio (1:100 or 1:50), the EAI of the phaseolin was nearly unaffected by the glycation with a 2.5 h incubation period (or with degree of glycation of about 40%), but significantly increased by the glycation, only with longer incubation periods, for example, 5−10 h (or with degree of glycation of ≥60%; Figure 6A). The improvement of the emulsifying activity by glycation with the same sugar or others has been similarly reported for other proteins, for example, soy glycinin[17] and pea vicilin,[15] but the extent of glycation applied is much less than that in the present work. For example, the EAI of pea vicilin was significantly improved by the glycation with

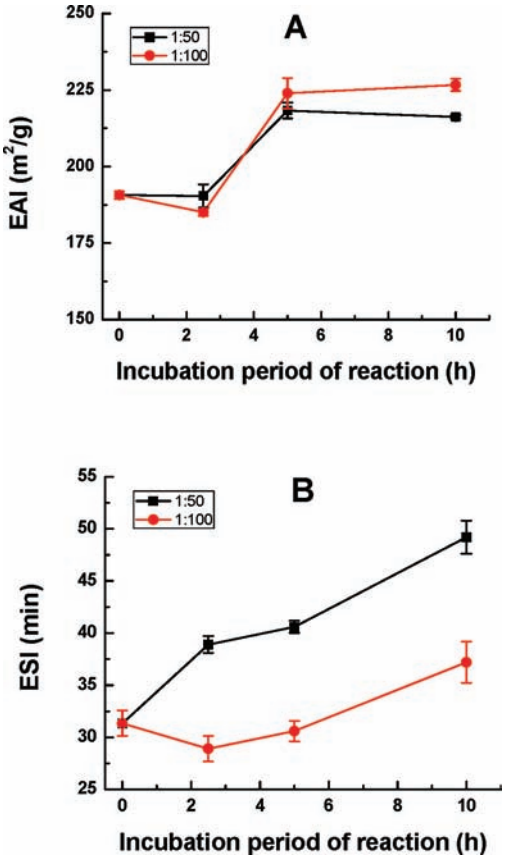

Figure 6. Emulsifying activity index (A) and emulsion stability index (B) of native and glycated phaseolin samples at pH 7.0. Each data point is the mean and standard deviation of at least three determinations.

glucose, lactose, galactose, and galacturonic acid, even at very low degrees of glycation (e.g., 4.7−13.4%[15]). However, in another previous work addressing pea legumin (11S globulin), glycation with galactose, at a degree of glycation of 37%, did not result in significant changes in its EAI.[14] Thus, the modification in emulsifying activity of 7S or 11S globulins by glycation is dependent on the nature of the proteins and the type and amount of applied sugar, as well as the degree of glycation.

In contrast with the EAI case, for which the change patterns of EAI upon increasing degree of glycation were similar at 1:100 and 1:50 molar ratios (Figure 6A), the influence of incubation period (or degree of glycation) on the ESI of phaseolin was heavily dependent on the applied molar ratio (Figure 6B). At 1:50 molar ratio, the ESI progressively increased from about 32 to 48 min, as the incubation period of glycation increased from 0 to 10.0 h, whereas at 1:100 molar ratio, the glycation with 2.5 h of incubation decreased the ESI, and after that, the ESI gradually increased with increasing incubation period. At 10.0 h of incubation, the ESI at the 1:100 molar ratio was still considerably lower than that of the 1:50 counterpart (Figure 6B). The diversity in the influence of glycation on the emulsion stability has been similarly observed in many previous papers.[14,15,17] For example, Baniel et al.[14] and Pedrosa et al.[15] indicated that the glycation resulted in an increase in the ability of pea 7S and 11S globulins to stabilize the emulsion, but Achouri et al.[17] pointed out that after extensive incubation of the reaction (e.g., >16 h), glycation resulted in a decrease in ESI of soy glycinin.

Structure−Emulsifying Functionality Relationships. In our previous work, we have found that the emulsifying activities (EAI

and ESI) of native vicilins are closely dependent on their surface hydrophilicity and/or hydrophobicity, for example, PS and H_0, as well as the flexibility in their tertiary and/or quaternary conformations.[9] These relationships seem to be also applicable in the glycated phaseolins. One interesting point to note is that the EAI was almost unaffected by glycation at 2.5 h of incubation; however, the treatment resulted in the highest extent of decrease in PS and, concomitantly, the highest extent of increase in H_0 (Table 1 and Figures 3 and 6A). As usual, increasing both PS and H_0 is beneficial for the EAI of the protein. Thus, it can be considered that at 2.5 h of incubation, the negative effect on the EAI, caused by the decrease in PS, was approximately comparable to the positive contribution from the increase in H_0.

In contrast, the ESI was greatly affected by glycation at 2.5 h of incubation, in an opposite way, depending on the applied protein/sugar molar ratio (Figure 6B). For example, the ESI was significantly improved at the 1:50 molar ratio, whereas at the 1:100 molar ratio, it on the contrary decreased a little. These observations clearly support our previous argument that the flexibility or stability in the tertiary conformation of vicilins plays a dominant role in emulsion stabilization,[9] because DSC, intrinsic fluorescence, and near-UV CD observations (Table 2 and Figures 4 and 5) had indicated distinct changes in tertiary conformations by the glycation. Herein, it is of interest to note that the glycation-induced tertiary conformational changes varied with the applied protein/sugar molar ratio, and at the 1:100 molar ratio, glycation resulted in a larger extent of tertiary conformational rearrangement than at the 1:50 molar ratio. Thus, the improvement of ESI by glycation at the 1:50 molar ratio can be largely attributed to an increase in flexibility in tertiary conformation, whereas the decrease in ESI at the 1:100 molar ratio may be due to increased stability in tertiary conformation, as a result of structural rearrangement.

The relationships between the conformations and the emulsifying activities can also be observed for the phaseolin glycated at extended incubation periods, for example, 5.0−10.0 h. As the incubation period of glycation increased from 2.5 to 5.0 or 10.0 h, both EAI and ESI were significantly improved by glycation (relative to the 2.5 h period; Figure 6). Although the improvement of PS at extended incubation periods (relative to the 2.5 h period; Figure 3) may be favorable for the improvement of the emulsifying properties (EAI and ESI), the contribution can be considered to be still limited, because the glycated phaseolin samples at 5.0 or 10 h periods exhibited much higher EAI, but their PS was still lower than that of the native counterpart. In these cases, the improvements of emulsifying properties, especially EAI, might thus largely result from progressive increases in the flexibility of quaternary conformation by glycation, as evidenced by the near-UV CD spectra (Figure 5B). This also supports our previous finding that the emulsifying activity of vicilins is closely related to the flexibility of their quaternary conformation.[9]

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