Bovine Serum Albumin-Sugar Conjugates through the Maillard Reaction: Effects on Interfacial Behavior and Emulsifying Ability

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Abstract: Bovine serum albumin (BSA) was employed as a model protein emulsifier to conjugate with aldohexose (α-glucose (Glc) or α-allose (All)) and sugar fatty acid ester (6-O-octanoyl-α-glucose (GlcC8)) through the Maillard reaction. It was found during the reaction that rate of decrease of free amino groups in BSA was almost the same for the BSA-sugar mixtures whereas browning and protein aggregation developed in the following order: Glc < All < GlcC8. It was thought that the rate of degradation of the Amadori compound could have been influenced by the OH-group stereochemistry at the C3 position of aldohexose, while denaturation of BSA by GlcC8 enhanced the browning and protein aggregation. To understand the emulsifying ability of the BSA-sugar conjugates, hexadecane-water interfacial tension and the oil droplet size of emulsions prepared by homogenizing hexadecane and aqueous solution of the conjugates were examined. BSA-GlcC8 showed greater improvement in interfacial and emulsifying activity than did BSA-Glc and -All. However, no improvement in emulsion stability was observed for any of the BSA-sugar conjugates, suggesting the weakness of the film formed at the oil droplet interface.

Key words: emulsifier, bovine serum albumin, Maillard reaction, interface tension, ζ-potential

1 INTRODUCTION

Proteins are widely used as emulsifiers in food, cosmetic, and pharmaceutical industries to homogenize insoluble liquids and maintain the homogeneous state of products1-3). To improve functional properties of proteins including their emulsifying ability, chemical modifications have often been made. Over the past few years, there has been growing interest in the modification of proteins with sugars through the Maillard reaction4-14); a complex network of non-enzymatic reactions between reducing sugars and protein amino groups (see Fig. 1). At an early stage of the Maillard reaction, amino groups in proteins react with carbonyl groups of sugars to give 1-amino-1-deoxy-ketose, known as the Amadori compound15). The set of reactions that occur thereafter results in the formation of both large protein aggregates and low molecular weight products that are believed to confer flavor, aroma, color, texture, and antioxidant characteristics to various foods. The reactivity of reducing sugars in the Maillard reaction was reported to decrease in the following order: aldopentoses > aldoketoses > disaccharides8,9,16), but little information is available on comparisons among the sugar isomers10).

It has been reported that products of the Maillard reaction exhibit improved emulsifying and foaming ability in some cases. To enhance the efficiency of globular proteins as an emulsifier, polysaccharides have often been used to covalently connect with proteins by the Maillard reaction4, 11-14). When the protein-polysaccharide conjugates adsorb at the oil-water interface in emulsion, polysaccharide chains will protrude into the aqueous bulk and provide a protecting layer that prevents the coalescence and flocculation of oil droplets. Wooster et al. closely investigated the effects of polysaccharides on the emulsion stability for β-lactoglobulin-dextran (or -maltodextrin) conjugates and confirmed that stability efficacy was a function of both thickness and density of the polysaccharide chains at the oil-water interface12,13). Another possible approach to improving the emulsifying ability of proteins is conjugation with alkyl chains17). Fatty acylated saccharides, having...
reducing sugar and fatty moiety, are the kinds of substances which can provide long alkyl chains on protein molecules via the Maillard reaction. It is expected that the conjugation of alkyl chains may facilitate protein adsorption at the oil-water interface and improve the packing state of adsorbed proteins to form a stronger barrier against the coalescence and flocculation of oil droplets.

In the present study, we investigated the impact of modification with bovine serum albumin (BSA) of aldohexose (D-glucose or D-allose) and of fatty acylated aldohexose (6-O-octanoyl-D-glucose) by the Maillard reaction (see Fig. 1). D-allose (All), an epimer of D-glucose (Glc), is not abundant in nature, but in recent years increasing interest in All has been directed towards its biological functions and possible utilization in medicines and foods. One of the objectives of this study was to compare the reactivity of Glc and All during the Maillard reaction and the emulsifying ability of the conjugates obtained. Another objective was to clarify the effect of conjugation with alkyl chains by the Maillard reaction on the protein emulsifying ability. BSA was employed as a model protein in this study since it contains many lysine residues and can be easily modified through the Maillard reaction. Also, the interfacial properties and emulsifying activity for native BSA have been well characterized and suitable experimental conditions for the emulsifying ability tests can be readily established. The BSA-sugar conjugates were prepared by dry-heating at a constant relative humidity and the emulsifying ability of conjugates was evaluated by measuring the oil droplet size in emulsions.

2 EXPERIMENTAL

2.1 Materials

Lyophilized bovine serum albumin (Fraction V, minimum 96%, product # A4503) was purchased from Sigma Chemical Co. (St. Louis, Mo.), stored in refrigerator at ca. 4°C, and used without further purification. D-glucose (Glc) was purchased from Tokyo Chemical Industry (Tokyo). D-allose (All) was kindly supplied from the Rare Sugar Research Center of Kagawa University. 6-O-octanoyl-D-glucose
(GlcC8) was synthesized via regioselective esterification of D-glucose, as explained in the following section. For the quantitative analysis of free amino groups of BSA-sugar conjugates, o-phthaldialdehyde (OPA) for biochemical studies was purchased from Wako Pure Chemical (Osaka). Hexadecane purchased from Wako Pure Chemical was used as the oil phase of emulsions. Reagents used to prepare aqueous buffer solutions were citric acid monohydrate, sodium acetate trihydrate, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium carbonate, and sodium hydrogen carbonate, all of which were guaranteed grade reagents from Wako Pure Chemical. Water was purified by passing through a Branstead E-pure purification system (Dubuque, IA).

2.2 Regioselective fatty acid esterification of D-glucose

6-O-octanoyl-D-glucose (GlcC8) was synthesized via regioselective esterification of D-glucose using octanoic acid vinyl ester as the acylating agent and lipase from Candida antarctica, Novozyme 435, immobilized on macroporous acrylic resin, as the catalyst. The advantage of the vinyl ester as an acylating agent is that the enol freed by transesterification is rapidly tautomerized into volatile acetaldehyde, thus making the process irreversible and simpler for product isolation. Briefly, D-glucose (180 mg) and octanoic acid vinyl ester (0.60 mL) were mixed in acetonitrile (2.3 mL) with Novozyme 435 (540 mg). The reaction mixture was stirred for 48 h at 45°C. Novozyme 435 was filtered off and washed twice with acetonitrile and ethyl acetate, respectively. GlcC8 was crystallized from the combined filtrate and further purified by recrystallization using ethyl acetate-hexane mixed solvent. Purity of the product was checked by TLC, reverse phase HPLC, and NMR.

2.3 Glycation of BSA by dry-heating

Covalent coupling between the amino groups of BSA and the reducing groups on carbohydrate by the Maillard reaction was achieved by dry-heating as follows. BSA and the sugar were dissolved in 10 mM sodium phosphate buffer (pH 7.0) with the molar ratio = 1:60 (weight ratio = 6:1 for sugar). The mixed solutions were freeze-dried, placed in a desiccator with saturated MgCl₂ solution (31% relative humidity), and then heated under reduced pressure at 50°C for a given incubation time. The incubated samples were dissolved in pure water to measure the absorbance by spectrophotometer (Hitachi U-1800) and to analyze the unreacted free amino groups by o-phthaldialdehyde (OPA) assay, for which native BSA was used as the standard. For these procedures, control experiments were also carried out by dry-heating BSA without adding any sugars.

2.4 Gel permeation chromatography

To evaluate the hydrodynamic size of BSA-sugar conjugates obtained through the Maillard reaction, gel permeation chromatography was performed using a Jasco LC-2000plus HPLC system equipped with TSKgel G3000PWXL column (10.7 mm ID × 30 cm, Tosoh, Co.) and UV-vis detector (Jasco UV-2070 plus). Sodium phosphate buffer (0.1 M, pH 6.9) containing 0.1 M NaCl was used as the eluent. A sample solution (1% (w/v), 20 µL) was applied to the column (30°C) at a flow rate 0.5 mL/min and the elution profile was monitored at 280 nm.

2.5 Interfacial tension

Interfacial tension between an aqueous solution of BSA-sugar conjugate and hexadecane was measured by means of the drop volume method employing an automated, computer-controlled apparatus (DVS-2000, Yashita Giken, Tokushima). This apparatus can precisely measure the volume of a pendant drop attached to a glass needle tip at a constant temperature with accuracy of ± 0.01°C. The sample solutions were sucked up in a glass syringe, then pushed out slowly to form a pendant drop in hexadecane. The maximum volume of the drop was determined from the detached drop volume estimated from the distance traveled by the piston inside the syringe of known inner diameter. Detachment of the drop was detected by an optical sensor. By controlling the speed of the moving piston, dynamic interfacial tension was obtained as a function of a pendant drop lifetime ranging from several minutes to two hours.

2.6 Preparation and characterization of emulsions

To prepare oil-in-water (O/W) emulsions, hexadecane was added to BSA-sugar conjugate solutions in 10 mM buffer of various pH and homogenized for 1 min using a sonicator (Branson sonifier 250) with the duty cycle set at 40%. The mean particle size of the emulsion was measured using a dynamic light scattering (DLS) instrument (ELS-8S, Osaka Electronics, Hirakata, Japan). To adjust the scattered light intensity to a level acceptable for the measurement, emulsions were diluted with the corresponding buffer and gently stirred just prior to the measurement.

By using the same DLS apparatus, but exchanging part of the optical system for electrophoretic dynamic light scattering, ζ-potential of each emulsion was measured. The electrophoretic mobility of oil droplets was estimated based on the laser-Doppler velocimetry, and the ζ-potential was calculated from Smoluchowski’s equation.

3 RESULTS AND DISCUSSION

3.1 Glycation of BSA by dry-heating

As an indicator of the progress of the Maillard reaction,

the development of a brown color in the BSA-sugar mixtures was monitored by measuring the absorbance for a 1% solution of each sample (Fig. 2A). Control BSA remained colorless throughout the heating time whereas browning of the BSA-sugar mixtures developed in the following order: Glc < All < GlcC8. Figure 2B shows, on the other hand, that the amount of unreacted amino groups of BSA in these three samples decreased at almost the same rate. The amount of free amino groups in BSA-Glc, BSA-All, and BSA-GlcC8 declined to about 50% in each after ca. 48 h incubation and then leveled off. This result suggests that the early stage of Maillard reaction proceeded at almost the same rate, but the reaction rate of successive processes, during which reactive small molecular intermediates and large protein aggregates are believed to be formed, depended on the structure of sugars. The elution profiles of gel permeation chromatography actually suggested different behaviors in the aggregation process for the three samples (see Fig. 3). Figure 3 shows that the elution pattern of control BSA, which originally contained a small amount of dimer, was not changed by heating for up to 70 h. For BSA-sugar mixtures, however, the main peak of monomeric BSA decreased with the heating time and new peaks of protein aggregates gradually emerged. We can see a correlation between the aggregation process and browning color development; that is, the more (less) browning of BSA-GlcC8 (BSA-Glc) that occurred, the more (less) aggregates contained.

In general, the major factors that influence the rate and extent of protein glycation are temperature and water activity as well as the nature and amount of the mixed reducing sugar. The initial kinetics of glycation are dependent on the proportion of the reducing sugar existing in the acyclic or active form under the reaction conditions and on the electrophilicity of the sugar carbonyl group. Our results suggested that the early stage of reaction (< 10 h) did not differ much between Glc and All but longer heating (> 48 h) resulted in more browning and aggregation for All than Glc. It is thought, therefore, that the OH-group stereochernistry at the C3 position of aldohexose influenced the degradation rate of the Amadori compound, leading to reactive small molecules and protein aggregation. It is interesting to note that the weight of mixtures decreased slightly after 48 h of heating; decreases of ~2.2%, ~3.5%, and 0% were observed for BSA-Glc, -All, and -GlcC8, respectively. This observation also suggested that the Amadori compound of BSA-All degraded faster and produced more volatile molecules than BSA-Glc. (At present, it is not clear why the weight for BSA-GlcC8 did not change during heating.) A greater extent of browning and protein aggregation by dry-heating (55°C, 65% relative humidity, 2 days) in All than in Glc was also reported for ovalbumin (OVA)-sugar mixtures, even though the amount of unreacted amino groups of OVA was comparable (ca. 40%) for both conjugates. In the case of α-lactalbumin-sugar mixtures, the reactivity of All was greater than that of Glc; the amount of unreacted amino groups was 43% (53%) in All (Glc) after 24 h dry-heating at 55°C and 55% relative humidity.

The results for BSA-GlcC8 in Fig. 2 and 3 showed more pronounced browning and protein aggregation compared with BSA-Glc and BSA-All. GlcC8 is a surface-active amphiphilic molecule with a critical micelle concentration of 26 mM (= 8.0 g/L) estimated by the drop volume method at 25°C (data not shown). It seems reasonable to infer that the three-dimensional structure of BSA became unstable by mixing GlcC8, although more common sugars like glucose usually stabilize protein structures. Preliminary differential scanning calorimetric experiments for BSA-GlcC8 showed a significantly decreased endothermic peak for the thermal denaturation of BSA, suggesting the destabilization of the BSA structure by GlcC8 (data not shown). It is
conceivable that denatured protein molecules have a different chemical reactivity and are more likely to aggregate during dry-heating. To prepare colorless BSA-sugar conjugates while avoiding aggregation, the heating time should be set at around 10 h and one can expect to obtain ca. 30% amino group modified BSA-sugar conjugates. From the viewpoint of application of the conjugates for foodstuffs, however, functionality such as flavor, aroma, color, texture, and antioxidant activity becomes important, and moderately aggregated brown colored conjugates may be more useful. We therefore used samples prepared by dry-heating for 48 h in the following experiments. (Note that heating too long, say 70 h, led to extended aggregation, especially for BSA-GlcC8, and quite low aqueous solubility of the conjugate.)

3.2 Oil-water interface tension

To understand the adsorption behavior of BSA-sugar conjugates, the interface tension, $\gamma$, between aqueous solution and hexadecane was measured as a function of drop lifetime. Figure 4 displays the adsorption kinetics for native BSA and BSA-sugar conjugates at various concentrations. A steady state of $\gamma$ was readily attained except in the low concentration solution (0.01 g/L), which showed a decrease in $\gamma$ in the early stage and leveling off after ca. 40 min. The steady state values of $\gamma$ have been plotted as a function of concentration in Fig. 5. We can see that values for $\gamma$ for BSA-Glc and -All were comparable to native BSA, but BSA-GlcC8 significantly lowered the interface tension, suggesting improved surface activity.

Some researchers have examined the adsorption behavior of conjugated proteins by measuring the air-water surface tension and reported that glycation of proteins by common monosaccharides only resulted in a small effect on the steady state surface tension. Trofimova et al. have confirmed that dynamic and equilibrium surface tensions of $\beta$-lactoglobulin-fructose conjugate solution (0.035 g/L) were comparable to those of native $\beta$-lactoglobulin. Interestingly, acetylated BSA showed faster adsorption to the air-water surface and increased molecular flexibility at the surface compared with native BSA. These results suggested that conjugation with a hydrophobic group rather than a hydrophilic sugar effectively improved the surface activity of proteins.

3.3 Emulsifying activity and emulsion stability

To understand the emulsifying ability of BSA-sugar conjugates, we prepared O/W emulsions homogenizing hexadecane and aqueous solution of the conjugates. First checked was the effect of oil content on emulsion size when native BSA was used as the emulsifier (see Fig. 6). The mean diameter of oil droplets was measured by DLS within 0.5 h after homogenization. When the oil content was set at 33% (v/v), the mean diameter was ca. 1.3 $\mu$m in the dispersion medium containing 5-10 g/L BSA and increased up to ca. 3 $\mu$m in dilute BSA (≤ 1 g/L) at which point the system became unstable. On reducing the oil content down to 4.8% (v/v), the droplet size became smaller than 1.3 $\mu$m except for the medium, 0.01 g/L BSA. This low oil content condition in the emulsifying activity tests
allowed the emulsifiers to be saved and was applied to the tests for BSA-sugar conjugates. Figure 6 shows that the droplet size of hexadecane emulsion stabilized by BSA-Glc and -All was slightly larger than that of BSA-stabilized emulsion, whereas BSA-GlcC8 clearly expressed a smaller diameter (0.5–1.1 μm) than BSA. These results were expectable, considering the surface activities of conjugates showed in Fig. 5. It can be seen in Fig. 5 that the modification of BSA by GlcC8 led to improved surface activity but that of Glc and All did not, and it is natural that only BSA-GlcC8 could form a smaller-sized O/W emulsion than native BSA. Note that the lowered interfacial tension is important to make small-size droplets during the droplet disruption of emulsification processes, but long-term stability of droplets after the emulsification can mainly be controlled by inter-particle forces (van der Waals, electrostatic, and solvation forces) and interfacial properties (fluctuation and mechanical strength) of the droplets.
Emulsification by BSA-sugar conjugates

Next, to characterize the electric state of emulsions, z-potential was measured as a function of pH. Figure 7 shows that no significantly different pH dependence was confirmed for the z-potential of emulsions stabilized by BSA or the BSA-sugar conjugates. At pH 6.7, the level at which the oil droplet diameter was evaluated in Figure 6, all emulsion samples indicated almost the same value (∼50 ± 5 mV). Around pH 4.5, which is close to the isoelectric point of native BSA (pH 4.7), z-potential was nearly zero, suggesting no net charge on the oil droplets and the emulsion stability presumably becoming quite low.

Finally, to confirm the stability of emulsions at pH 6.7, we examined the time course of droplet diameter (Figure 8). It can be seen in Figure 8 that within hours after homogenization, BSA-Glc and -All showed significant increases in droplet size. The droplet size for BSA-GlcC8 also steadily increased although the initial diameter was smaller than that of BSA and the other conjugates. This result suggests that the conjugation of BSA with GlcC8 was not effective in improving emulsion stability although it was effective in promoting surface activity.

It is generally thought that the stabilization of oil droplets in protein solution derives from the protein film formed at the oil-water interface that provides electrostatic and steric repulsive forces between droplets. In the present situation, the electrostatic contribution was thought to be the same for BSA and BSA-sugar conjugates, since they showed the same z-potential value at pH 6.7. The low emulsion stability for BSA-sugar conjugates may be explained as the decreased steric repulsion of interfacial protein film. It should be noted that modification of BSA by monosaccharides often increased molecular flexibility at the surface compared with the native BSA, and it is conceivable that the molecular flexibility correlates to the mechanical strength of the film. A mechanically weak interface film will easily be broken during collisions between oil droplets, resulting in accelerating coalescence and droplet size increment, as shown in Figure 8.

It is interesting to note that Takahashi et al. reported that lysozyme conjugated by glucose stearic acid by the Maillard reaction led to the improvement of both emulsifying activity and emulsion stability whereas acylation of lysozyme with N-hydroxysuccinimide ester of palmitic acid improved only the emulsifying activity but not emulsion stability as was observed for BSA-GlcC8 in this study. Consequently, it is concluded that the effects of conjugation of proteins with aliphatic chains to the emulsion stability are influenced by many factors such as the protein type, hydrocarbon chain length, and the presence or absence of glucosidic spacers between the protein surface and aliphatic chain.

4 CONCLUSION

For the Maillard reaction of BSA with Glc, All, and GlcC8, it was confirmed that the decreasing rate of free amino groups of BSA was almost the same, but browning...
and protein aggregation developed differently in the order Glc < All < GlcC8 during dry-heating. It is thought the OH-group stereochemistry at the C3 position of aldohexose influenced the degradation rate of the Amadori compound, while denaturation of BSA by GlcC8 enhanced the browning and protein aggregation processes. The interfacial and emulsifying activities of the conjugates for both BSA-Glc and -All were comparable with (or slightly worse than) native BSA whereas BSA-GlcC8 showed improvement. Therefore, the use of fatty acid sugar ester for the modification of proteins through the Maillard reaction seems advantageous for improving the functionalities of proteins. Disappointingly, however, no improvement in the emulsion stability for BSA-GlcC8 was observed, suggesting weakness of the interfacial film at the oil droplet interface formed. It may be expected that the conjugation with sugar ester of a longer-chain fatty acid, for example stearic acid, or the simultaneous conjugation with both sugar ester and polysaccharide will improve the emulsion stability by forming a stronger or thicker interfacial film.

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