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Browning and Protein Polymerization Induced by Amino–Carbonyl Reaction of Ovalbumin with Glucose and Lactose

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Ovalbumin (OVA) was stored with glucose or lactose in a solid system. Though there was no major difference in the decrease of the free amino group between OVAs stored with glucose and lactose, the browning of the OVA–glucose system was much stronger than that of the OVA–lactose system. Removal of free glucose in the early storage of the reaction strongly suppressed the browning reaction, while the addition of glucose to lactosylated OVA promoted it. Glucose induced protein polymerization more strongly than lactose, and the polymerization proceeded even after free glucose was removed during storage. These results suggested that the presence of free glucose in the storage system was an important factor for browning but not for protein polymerization and that the Amadori rearrangement compound formed in the OVA–glucose system was smoothly converted into aldehyde compounds; however, the Amadori compounds formed in OVA–lactose and 4-*O*-methyl-D-glucose systems were stabilized by the protecting effect of the galactopyranoside and methyl group, respectively, on 4-OH of glucopyranosides.

Browning, protein insolubilization, and reduction of protein nutritional value are well-known to result from the protein–sugar Maillard reaction. The glycosylation was studied on lysine residues in protein such as RNase (Baynes et al., 1984) and collagen (Monnier et al., 1984). However, there are only a few reports comparing the Maillard reactions of various sugars with protein amino groups. Bunn and Higgins (1981) reported that the rate of Schiff base formation of various sugars with protein under physiological conditions depended on the extent of carbonyl formation rather than the ring structure. We previously reported that in a solid system a decrease in protein amino group induced by the reaction with glucose was not so different from that induced by the reaction with galactose, whereas browning and protein polymerization of the protein–galactose system proceeded more strongly than that of protein–glucose system (Kato et al., 1986).

Lactose is a major sugar in milk, and the Maillard reaction in some dairy products has been investigated from nutritional and biochemical viewpoints (Möller, 1981; Lee et al., 1979). The studies evaluating the Maillard reaction during processing and storage in lactose-hydrolyzed milk products induced much greater protein quality losses than in unhydrolyzed milk. This difference might be attributed to the difference in Maillard reaction between glucose–galactose–protein and lactose–protein systems (Burvall et al., 1977). However, the comparison of the reactivities of lactose and glucose with protein have not yet been investigated under the same condition.

The experiments reported here were designed to investigate browning and polymerization of protein occurring during Maillard reaction of ovalbumin–glucose and ovalbumin–lactose systems.

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MATERIALS AND METHODS

Preparation of Maillard-Reacted Protein. Ovalbumin (OVA) was prepared from fresh egg white of White Leghorn hens by the ammonium sulfate method (Marshall and Neuberger, 1972). Glucose and lactose were purchased from Waco Co., Ltd. (reagents of superfine grade).

The mixtures of ovalbumin and glucose (OVA–glu) (1:1, w/w) or lactose (OVA–lac) (1:2, w/w) were dissolved in distilled water, and the solution was adjusted to pH 7.5 with dilute NaOH. These mixtures were freeze-dried and kept in desiccators for various periods (0–26 days) at 50 °C and 65% relative humidity (RH) maintained with saturated KI solution. As controls, ovalbumin and each sugar were individually maintained for 26 days in a similar manner.

Separation of Free Sugars from OVA–Sugar Systems. Two-day-stored OVA–glu and 4-day-stored OVA–lac samples in which protein amino groups were blocked at almost the same rate were filtrated with Sephadex G-50, and free sugars were removed from the ovalbumin–sugar mixtures. These protein–sugar adducts without free sugars were adjusted to pH 7.5 with dilute NaOH solution. The freeze-dried samples were stored for another 1–3 weeks at 50 °C and 65% RH. Ovalbumin–glucose or –lactose complexes were abbreviated as OVA–G and OVA–L, respectively.

Electrophoresis. Sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis (8% acrylamide) (SDS-PAGE) was performed by the method of Laemmli (1970).

Browning. Browning of OVA–sugar mixtures (1 mg/mL protein concentration) was measured by absorbance at 420 nm. The insoluble protein–sugar mixtures were solubilized by hydrolysis with Nagase (2 Penn Plaza, New York, NY) for 2 h at 37 °C, and the absorbance was recorded.

Analytical Methods. Free amino groups were measured by the fluorometric method using fluorescamin (Rade) according to Böhlen et al. (1973). The fluorescence

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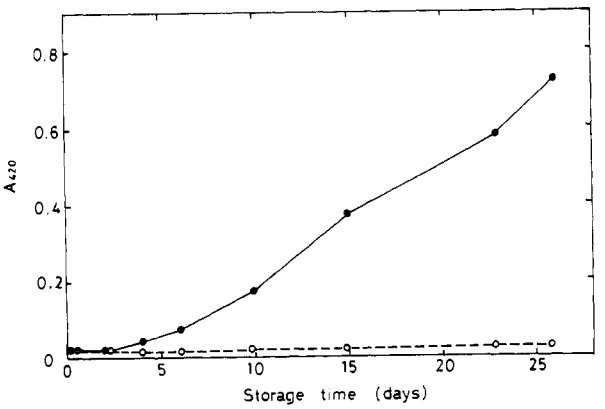

Figure 1. Browning of ovalbumin–glucose (●) and –lactose (○) mixtures during storage for 26 days at 50 °C and 65% RH.

was determined on a Jasco spectrofluorometer FP-550 with the excitation at 390 nm and emission at 485 nm.

Amino acid analyses were performed with Type JLC-6AH amino acid analyzer (JEOL, Tokyo) after hydrolysis at 110 °C for 24 h in 6 N HCl in an evacuated, sealed tube.

Synthesis of 4-*O*-Methyl-D-glucose. 4-*O*-Methyl-D-glucose was derived by hydrolysis of 4-*O*-methyl-α-methyl-D-glucose by the method of Smith (1951). Methyl 4-*O*-methyl-α-D-glucoside was prepared from methyl α-D-glucoside 2,3,6-tribenzoate by treatment with methyl iodide–silver oxide followed by treatment with sodium methoxide by the modified methods of Levene and Raymond (1932), Evans (1972), and Horton and Weckerle (1975).

RESULTS AND DISCUSSION

Browning and protein polymerization are usually observed at the late stage of the Maillard reaction of a protein–sugar system. The reactivity of ovalbumin with a monosaccharide such as glucose was compared with that of lactose, a disaccharide. The browning profiles of OVA–glu and OVA–lac during storage for 26 days are shown in Figure 1. The brown coloring of OVA–glu increased gradually with storage. However, OVA–lac gave almost no color even after 26-day storage. Thus, the browning reaction of glucose system was remarkably stronger than that of the lactose system.

To determine whether such a remarkable difference in degree of browning between OVA–glu and OVA–lac systems was due to a difference in the rate of condensation reaction between sugar carbonyl and protein amino groups, the free amino group content of OVA stored with glucose or lactose for 26 days was determined (Figure 2). The remaining amino groups were expressed as the relative value (%) to the free amino groups of native ovalbumin based on fluorometric intensities. The free amino group contents of OVA–glu and OVA–lac decreased to 20 and 26%, respectively, after storage for 26 days. The storage time to attain 50% blocking of amino group were about 2 and 4 days for OVA–glu and OVA–lac, respectively. Though there were slight differences in amino group decreases between the two OVA–sugar systems described above, the remarkable difference in browning between the two systems (Figure 1) could not be adequately explained by such a slight difference in the amino group decreases.

The effect unreacted free or condensed sugars on browning of the protein–sugar systems was investigated by separating free sugars from the systems followed by restorage. The free glucose and free lactose were removed from respective systems after 2- and 4-day storage, respectively. About 50% of the OVA amino groups had reacted with each sugar in both OVA–glu or OVA–lac

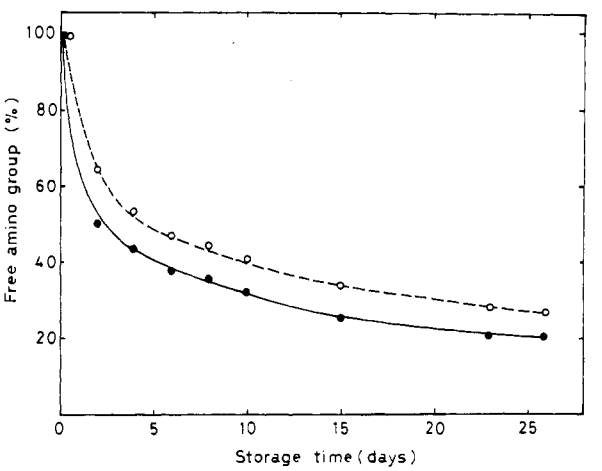

Figure 2. Decrease in the free amino group of ovalbumin during storage with glucose (●) or lactose (○) for 26 days.

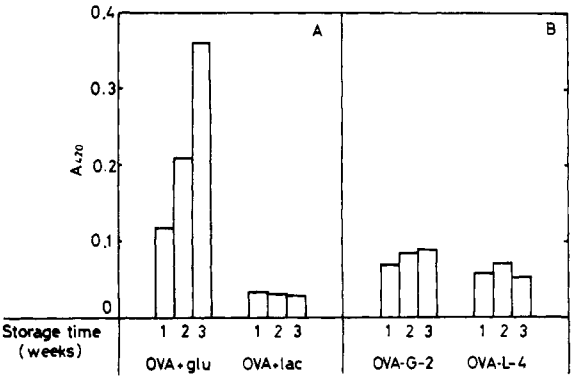

Figure 3. Suppression of browning by eliminating free glucose from the OVA–glucose system. OVAs reacted with sugars (OVA–G-2, OVA–L-4) were stored another 1–3 weeks after eliminating free sugars (B). Browning of OVAs stored in the presence of free sugars is also indicated for comparison (A).

systems (Figure 2). The protein–sugar complexes were then stored for another 1–3 weeks, and the brown coloring of each sample was monitored by UV during storage. As shown in Figure 3, removal of unreacted free glucose from the OVA–glu system strongly suppressed the browning reaction. This suggests that free glucose in the storage system played an important role in the browning reaction.

To confirm the contribution of free sugar to the browning reaction, glucose or lactose was added again to the OVA–sugar systems of which free sugars had been removed after 2-day (OVA–glu) or 4-day (OVA–lac) storage, and the brown coloring of each sample were monitored for an additional 3 weeks. As shown in Figure 4, the addition of glucose strongly promoted the browning reaction not only for OVA–glucose but also for the OVA–lactose, whereas addition of lactose induced only a slight browning even for OVA–glu. These results indicated that free glucose but not free lactose was an essential factor for browning reaction of the OVA–sugar systems; however, the reason why lactose had no effect on browning is still unclear.

The protein polymerization induced by Maillard reaction was analyzed by SDS–polyacrylamide gel electrophoresis (Figure 5). As the storage period increased, the electrophoretic mobility and the band intensity of OVA decreased gradually and protein bands with small mobility corresponding to polymerized OVA appeared. These electrophoretic changes were more marked for OVA–glu than for OVA–lac, suggesting that glucose strongly accelerated not only the browning reaction but also protein

Table I. Evaluation of Fructosyl- or Lactulosyllysine and Reactive Lysine of Maillard-Reacted Ovalbumin Stored for 7 Days with or without Free Sugars as Measured by Amino Acid Analysis after Acid Hydrolysis

	control					
mol/mol of protein	OVA–G-2	OVA–L-4	OVA–G-2[a]	OVA–L-4[a]	OVA–glu-2[b]	OVA–lac-4[b]
lysine[c]	14.6	16.2	12.5	16.4	11.1	16.2
furosine[c]	1.7	2.0	1.9	1.9	2.3	2.2
reactive lysine[d] (free lysine + Schiff base)	10.3	13.7	7.9	14.0	5.5	13.5
fructosyllysine[e]	8.6		9.3		11.2	
lactulosyllysine[f]		6.2		5.9		6.7

[a] The samples were stored 7 days after free sugars were removed by gel filtration from the OVA–glucose system stored for 2 days and the OVA–lactose system stored for 4 days. [b] The protein–sugar mixtures stored for 2 days (OVA–glu-2) or 4 days (OVA–lac-4) were stored for another 7 days in the presence of free sugars (glucose or lactose). [c] Determination by amino acid analysis. [d] [Reactive lysine] = [free lysine] + [Schiff base] = [lysine] − 0.5[fructosyllysine] or [lysine] − 0.4[lactulosyllysine]. [e] [Fructosyllysine] = 4.9[furosine]. [f] [Lactulosyllysine] = 3.1[furosine].

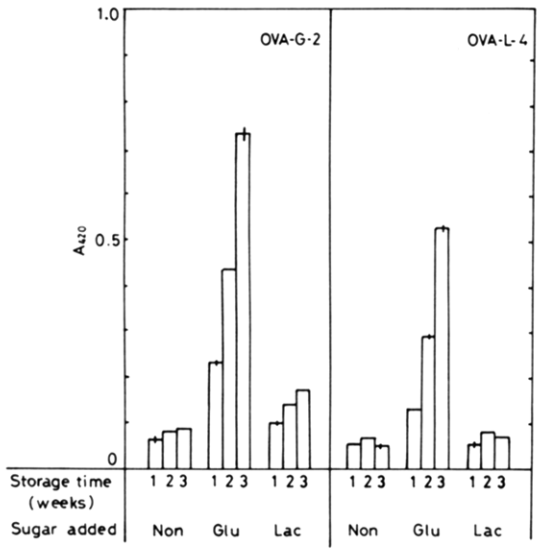

Figure 4. Effect of free sugars on the browning of OVA–G and OVA–L. The free sugars were eliminated from OVA–sugar mixtures after 2- or 4-day storage (OVA–G-2, OVA–L-4), and the OVA–sugar complexes were then stored for another 3 weeks with or without free sugars (glucose or lactose).

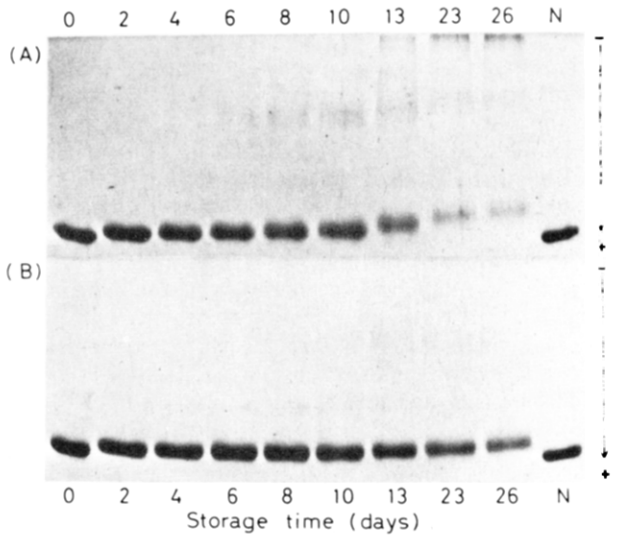

Figure 5. Protein polymerization during the storage of OVA with glucose (A) or lactose (B) for 26 days as measured by SDS-PAGE. N: native ovalbumin.

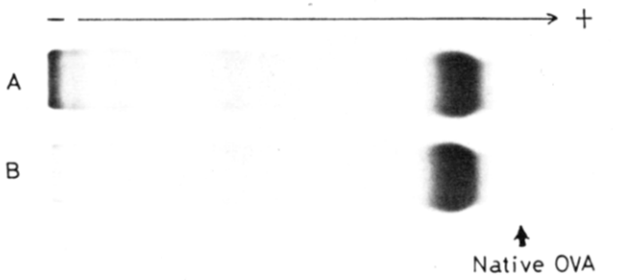

Figure 6. Effect of free sugars on protein polymerization during storage as measured by SDS-PAGE. OVAs reacted with sugars (A, OVA–G-2; B, OVA–L-4) were stored another 7 days after elimination of free sugars.

polymerization than lactose.

The effect of free sugar on protein polymerization during amino–carbonyl reaction was also analyzed by SDS-PAGE for the samples without free sugars described above (see Figure 3). As shown in Figure 6, protein bands corresponding to high molecular size polymers with low electrophoretic mobility were observed for the OVA–G system, whereas almost no polymers were detected for the OVA–L system. This result indicates that protein–sugar adducts formed in the early stage of the Maillard reaction are polymerizing quite independently of the presence of free sugar and that protein-bound glucose adducts promoted protein polymerization more strongly than protein-bound lactose adducts. Because the reaction rate of glucose binding to protein amino groups was not much larger than that of lactose binding, the accelerated polymerization of the glucose system might be due to rapid degradation of the Amadori compound fructosyllysine into Maillard reaction products that have high reactivity to other molecules.

The contents of Amadori rearrangement compounds, fructosyllysine and lactulosyllysine, of OVA–G and OVA–L stored for 9–11 days with or without free sugars were quantitated by the determination of furosine after hydrolysis according to the methods of Bujard and Finot (1978) and Finot et al. (1981). The contents of the Amadori rearrangement compounds, that is fructosyllysine formed in the glucose system and lactulosyllysine formed in the lactose system, were compared in Table I. The content of Amadori compound (fructosyllysine) in the glucose system was about twice as much as that of Amadori compound (lactulosyllysine) in the lactose system, either with or without free sugars. On the other hand, the contents of free lysine plus Schiff base in the glucose systems were lower than those of the lactose systems. These results suggested that Amadori rearrangement in the glucose system occurred more easily than that of the lactose system. The Amadori compounds in glucose systems as comparing with a control, OVA–G-2, slightly increased by further storage for 1 week. These facts indicated that the protein-bound glucose adducts were gradually changed to other compounds during storage.

Browning and polymerization proceeded through a further reaction of Amadori rearrangement compounds.

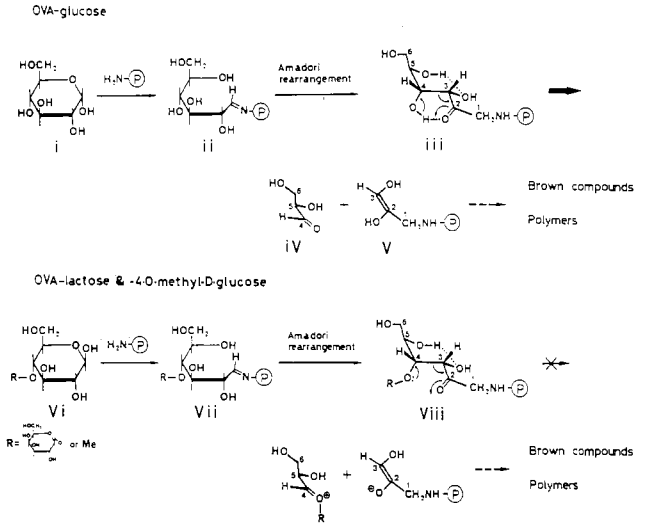

Figure 7. Schematic diagram showing the stabilization of lactose– and 4-*O*-methyl-D-glucose–protein Amadori compounds; comparison with the stability of the glucose–protein Amadori compound.

Ovalbumin–glucose was converted into browning products via the concerted cleavage mechanism of Amadori rearrangement compounds (Kato et al., 1986). On the other hand, in the ovalbumin–lactose system, it was converted very slowly into browning products (Figures 1 and 3), though protein amino groups decreased nearly as much as in the OVA–glucose system. These results indicated that lactose led to an Amadori rearrangement compound by reaction with the amino group of ovalbumin, but the Amadori compound was difficult to degrade into aldehyde compounds. Since the sugar moiety of lactose reacting with the amino group is glucopyranoside, the rearrangement mechanism from (i) to (iii) and (vi) to (viii) may be the same in both systems. The Amadori compound from ovalbumin–glucose formed an intermediate, two six-membered rings with a cis juncture, which is stabilized by two hydrogen bondings between 3-OH and 5-OH and C2-carbonyl and 4-OH, and then cleaved between the C3–C4 bond by a concerted mechanism (Figure 7, iii). The resulting aldehyde derivatives (iv and v) may be key intermediates to formation of brown compounds. However, the Amadori rearrangement compound derived from the ovalbumin–lactose system is only possible by a hydrogen bonding between 3-OH and 5-OH groups; it is impossible to take a similar concerted cleavage mechanism for the C3–C4 bond fission (Figure 7, viii).

It is indicated that the galactopyranoside group in the Amadori rearrangement compound acts as a protection group of the 4-OH group, and the free hydroxy group in the compound is a definitive factor for further degradation into browning compound.

This fact was confirmed on the storage experiment of 4-*O*-methyl-D-glucose and ovalbumin. This ovalbumin–4-*O*-methyl-D-glucose system was stable, as well as the ovalbumin–lactose system, and did not show entire browning after 2-week storage (Figure 8). The ovalbumin–lactose system also did not exhibit browning after 2-week storage. These facts showed that the C3–C4 bond fission became difficult not only by methylation at the

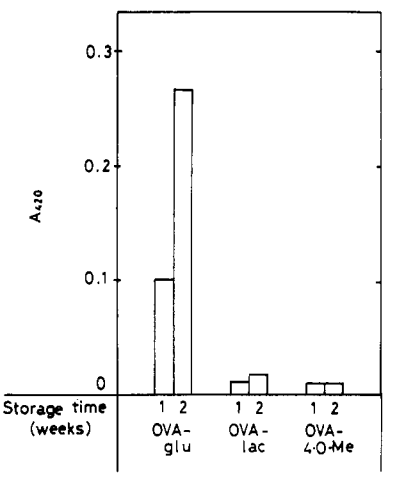

Figure 8. Browning of ovalbumin–4-*O*-methyl-D-glucose, –glucose, and –lactose mixtures during storage for 2 weeks at 50 °C and 65% RH.

4-OH group but also by acetal bonding as lactose. We do not conclude that this degradation mechanism adapts to amino–carbonyl reactions under various conditions. In other systems and under specific conditions, browning reaction may proceed via other pathways with intermediates, e.g., 3-deoxyglucosone (Hodge, 1953). Effects of substituent on hydroxy groups other than the 4-OH group, the major role of the free glucose in browning reactions, and substituent effects on sugars in other systems under various conditions will be explored in further studies.

Registry No. Glucose, 50-99-7; lactose, 63-42-3; 4-*O*-methyl-D-glucose, 4132-38-1; ε-fructosyllysine, 21291-40-7; lactulosyllysine, 34326-63-1.

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