Effect of Some Metals on the Maillard Reaction of Ovalbumin

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Studies were conducted to investigate the effect of metal ions such as Na^+, Cu^{2+}, and Fe^{3+} or Fe^{2+} on the Maillard reaction during storage (50 °C; 65% relative humidity) of freeze-dried ovalbumin–glucose mixtures and the changes of some ovalbumin properties ascribed to the reaction. Cupric ion and Fe^{3+} accelerated the denaturation (the decrease in α-helix content) of ovalbumin by promoting the Maillard reaction which was ascertained from the browning color development, the increase and decrease in solubility, the variation in isoelectric point determined by isoelectric focusing, the destruction of lysine and arginine residues, and the remaining ratios of free amino groups. However, Na^+ had no effect on the reaction. The browning reaction was promoted somewhat faster in the presence of Fe^{3+} than of Fe^{2+}. The addition of increasing amounts of Cu^{2+} ion from 0 to 0.5 mg % resulted in the acceleration of the browning reaction and the denaturation of ovalbumin.

The interaction of metallic ions with proteins has been studied extensively in its biochemical as well as physicochemical aspects (Vallee and Wacker, 1970). As one of the subjects of the metal–protein interaction, the significance of metals for the Maillard reaction has also been recognized because of its acceleration or inhibition effect on the reaction between protein or amino acid and sugar in terms of the amount and type of metal (Ellis, 1959).

In our earlier studies (Kato et al., 1978; Watanabe et al., 1980), the browning reaction in the dried egg white solid–glucose system was found to be promoted more remarkably than in the dried ovalbumin–glucose one. We also suggested that trace amounts of metal in egg white accelerated the rate of browning. The present study was designed to investigate the effect of metal ions such as Na^+, Cu^{2+}, and Fe^{3+} or Fe^{2+}, which were generally present in egg white, on the rate of the Maillard reaction in the dried ovalbumin–glucose system and the change of some properties of ovalbumin ascribed to the reaction.

MATERIALS AND METHODS

Sample Preparation. The ovalbumin preparation was dissolved with water at the protein concentration of 1% and divided into two parts. One part was adjusted to pH 10.0 and freeze-dried. This sample was termed OV, as described in a previous study by Watanabe et al. (1980). To the several divided solutions of the other part, some metal ions were added with D-glucose corresponding to 30% of the dry weight of the protein. The rates of the metal ion additions were as follows: $FeCl_2$, $FeCl_3$, and their mixture in equal amounts at the rate of 5 mg % to compare the effect of oxidative or reductive forms of iron, and NaCl at the rate of 0.2% and $CuSO_4$ and $Fe(NO_3)_3$ at the rate of 5 mg % to evaluate the effect of the type of metal. These solutions were adjusted to pH 10.0 and then freeze-dried. Samples containing $FeCl_2$, $FeCl_3$, $FeCl_2$ + $FeCl_3$, NaCl, $CuSO_4$, $Fe(NO_3)_3$, and with no metal were designated OVG–Fe^{2+}, OVG–Fe^{3+}, OVG–(Fe^{2+} + Fe^{3+}), OVG–Na, OVG–Cu, OVG–Fe, and OVG, respectively. The various samples containing Cu^{2+} at the rate of 0.001 mg % (OVG–Cu-I), 0.005 mg % (OVG–Cu-II), 0.05 mg % (OVG–Cu-III), and 0.5 mg % (OVG–Cu-IV) for the OVG solutions were prepared by the same method as mentioned above. For the control, an OV–Cu system containing $CuSO_4$ at the rate of 0.5 mg % (OV–Cu-IV) was treated in the same manner.

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These samples were stored at 50 °C and 65% relative humidity as described previously (Kato et al., 1978).

Measurement of Brown Color, Solubility, Amino Acid Composition, and α-Helix Content. All methods used for the measurements of brown color, solubility, amino acid composition and α-helix content were the same as reported in a previous study by Watanabe et al. (1980).

Determination of Free Amino Groups. The content of free amino groups was determined by the method of fluorometric assay (Böhlen et al., 1973), using a Hitachi fluorescence spectrophotometer, MPF-2A, with the excitation wavelength at 390 nm and emission at 475 nm. The remaining ratios of free amino groups were expressed as percentages in relation to the content of free amino groups in native ovalbumin.

Isoelectric Focusing. The isoelectric points of the unstored and stored samples with or without metal ions were determined by isoelectric focusing in 5% polyacrylamide gel, using the technique based on the method of Catsimpoolas (1968). Each sample was dissolved in pH 7.0 phosphate buffer (I = 0.01) at the rate of 5% and focused in polyacrylamide gel containing 2% ampholine (pH range of ampholine: unstored sample, pH 3.5–10.0; stored sample, pH 3.0–5.0) for 4 h at 200 V. Each point on the pH gradient represents the average pH value obtained when duplicate unstained gels were cut into 0.5-cm slices and eluted with distilled water for one night. Proteins were fixed with 12.5% trichloroacetic acid and stained with Coomassie brilliant blue G-250. A Shimadzu dual-wavelength scanner, Model CS-910, was used to obtained the absorbance migration tracings.

RESULTS

Effect of the Added Ferrous and Ferric Ions. The browning color development on the addition of Fe^{2+} and Fe^{3+} ions is shown in Figure 1. From the comparison of four lines in Figure 1, it was found that the browning was positively catalyzed by iron ions and that the Fe^{3+} ion accelerated the color development reaction somewhat faster than that observed in the presence of the Fe^{2+} ion.

Effect of the Added Three Metallic Ions. The shape of the browning curve on the addition of three metallic ions is shown in Figure 2. The browning color in the OVG–Cu system developed from its initial storage time and showed ~4 times higher optical density than the others after 2 days of storage. That in the OVG–Fe system developed gradually after storage for 2 days. The lines on the OVG and OVG–Na systems were the same and almost linear with storage time.

Figure 3 shows the changes in solubility which occurred in the stored samples. Solubilities of OVG and OVG–Na

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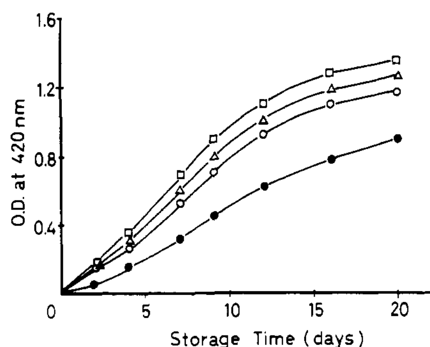

Figure 1. Effect of Fe^{2+} and Fe^{3+} additions on the browning color development in ovalbumin-glucose mixtures. (●) OVG; (○) OVG–Fe^{2+}; (□) OVG–Fe^{3+}; (△) OVG–(Fe^{2+} + Fe^{3+}).

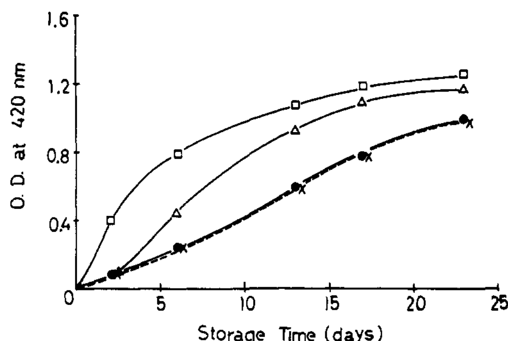

Figure 2. Effect of Na^{+}, Cu^{2+}, and Fe^{3+} additions on the browning color development in ovalbumin-glucose mixtures. (●) OVG; (×) OVG–Na; (□) OVG–Cu; (△) OVG–Fe.

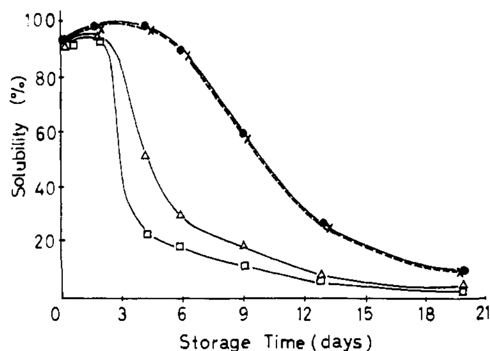

Figure 3. Effect of Na^{+}, Cu^{2+}, and Fe^{3+} additions on the change of solubility in ovalbumin-glucose mixtures. (●) OVG; (×) OVG–Na; (□) OVG–Cu; (△) OVG–Fe.

during storage for the 5 days were much better than those of unstored samples, as noted in the OVG system in a previous study by Watanabe et al. (1980). Moreover, these curves for both samples showed almost the same pattern throughout the storage period. On the other hand, solubilities of OVG–Fe and OVG–Cu began to decrease abruptly after 2 days of storage, followed by a more moderate decrease. As seen in Figure 3, OVG–Cu had the lowest solubility, which decreased to 25% within the first 4 days.

The remaining ratios of basic amino acid during the storage obtained from the amino acid composition are shown in Table I. The destruction of lysine and arginine residures was remarkable, and their remaining ratios reached 60% in the first 3 days of storage, followed by only slight decreases during the 3 days to follow. However, there was no destruction of histidine residue in all samples used.

The changes in free amino group content occurring during storage shown in Figure 4 indicated that the losses of free amino groups were similar in extent among all kinds of samples, reaching ~70% in the storage for the first day, followed by a slow increase. This was similar to the phenomenon encountered in the destruction of lysine (Table I).

Each isoelectric point of various peaks separated by isoelectric focusing in polyacrylamide gel columns on the unstored and stored samples is shown in Figure 5. Isoelectric points on the unstored OVG–Cu and OVG–Fe showed only little differences for OVG and OVG–Na samples in the presence of compounds which have a more acidic isoelectric point than native ovalbumin. The major observable change with the samples stored for 3 days was the disappearance of the native ovalbumin and the occurrence of various compounds having more acidic isoelectric points, in the order of OVG–Cu, OVG–Fe, OVG–Na, and OVG, based on the changes in net charge from the blocking of basic amino groups.

Table I. Effect of Na^{+}, Cu^{2+}, and Fe^{3+} Additions on the Remaining Ratio of Basic Amino Acid Residues in Ovalbumin-Glucose Mixtures

basic amino acid	days	remaining ratio of basic amino acid residues			
		OVG	OVG-Na	OVG-Cu	OVG-Fe
His	3	100	100	100	100
	6	100	100	100	100
Lys	3	59	59	57	58
	6	58	58	56	56
Arg	3	60	60	58	59
	6	59	59	51	56

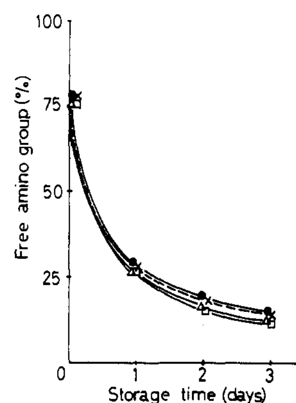

Figure 4. Effect of Na^{+}, Cu^{2+}, and Fe^{3+} additions on the change of the free amino group content in ovalbumin-glucose mixtures. (●) OVG; (×) OVG–Na; (□) OVG–Cu; (△) OVG–Fe.

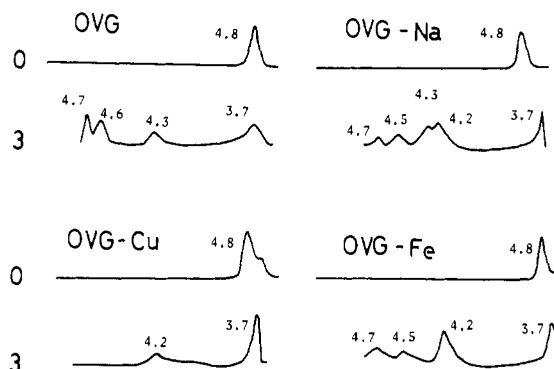

Figure 5. Effect of Na^{+}, Cu^{2+}, and Fe^{3+} additions on the change in the isoelectric point in ovalbumin-glucose mixtures. Storage time: 0, without storage; 3, 3 days of storage. pH range of ampholine used: unstored sample, pH 3.5–10.0; stored sample, pH 3.0–5.0.

Table II. Effect of Na^+, Cu^{2+}, and Fe^{3+} Additions on the Loss of α-Helix Content in Ovalbumin-Glucose Mixtures

	α-helix content, %, at storage time, days, of				
	0	3	6	12	20
OVG	33	31	29	27	19
OVG-Na	33	29	28	25	14
OVG-Cu	33	21	17	14	6
OVG-Fe	33	26	22	18	12

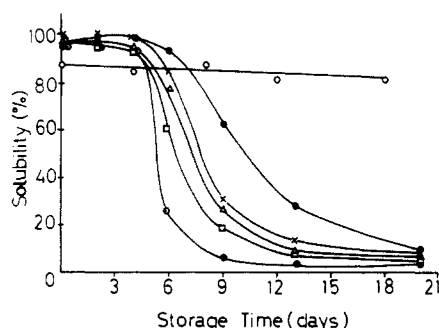

Figure 6. Effect of the concentration of added Cu^{2+} on the change of solubility in ovalbumin-glucose mixtures. (●) OVG; (×) OVG-Cu-I; (△) OVG-Cu-II; (□) OVG-Cu-III; (⊙) OVG-Cu-IV; (○) OV-Cu-IV.

The variation in the α-helix content of the ovalbumin calculated from the CD curves of the soluble part (pH 7.0 phosphate buffer, $I = 0.1$) of unstored and stored samples is shown in Table II. Watanabe et al. (1980) reported that native ovalbumin contained an α helix of 33%. The α-helix contents of the four unstored kinds of samples were ~33% and did not show changes in secondary form during their preparation. However, the α-helix content gradually decreased with storage time, showing that Cu^{2+} and Fe^{3+} ions with the exception of Na^+ accelerated the destruction of the ovalbumin secondary structure.

Effect of Cupric Ion Concentrations. Solubilities of the unstored and stored samples containing $CuSO_4$ in various concentrations are shown in Figure 6. For the first 4 days, there were only marginal differences in the solubilities of all samples of OVG system, but thereafter their solubilities seriously decreased with the addition of increasing amounts of Cu^{2+} ion from 0 to 0.5 mg %. In contrast, the solubilities of the OV-Cu-IV containing no glucose showed a slight decrease during the storage time studied. From the fact that 0.023 mg % of copper ion is present in edible egg white liquid (Cotterill et al., 1977), it was expected that the browning curve of OVG containing the amount of copper ion present in egg white would be situated between the curve of OVG-Cu-I and that of OVG-Cu-II.

The α-helix content of ovalbumin also decreased with the increase of Cu^{2+} ion concentration and the period of storage time, as shown in Table III. However, there was no change in the α-helix content in the samples of OV-Cu-IV. This is in contrast to the decrease in the secondary structure for the OVG system.

Table III. Effect of the Concentration of Added Cu^{2+} on the Loss of α Helix in Ovalbumin-Glucose Mixtures[a]

	α-helix content, %, at storage time, days, of		
	0	3	6
OVG	33	31	29
OVG-Cu-I	33	31	27
OVG-Cu-II	33	29	26
OVG-Cu-III	33	28	25
OVG-Cu-IV	33	28	22
OV-Cu-IV	33	33	33

[a] I, +0.001 mg % $CuSO_4$; II, +0.005 mg % $CuSO_4$; III, +0.05 mg % $CuSO_4$; IV, +0.5 mg % $CuSO_4$.

DISCUSSION

The results reported here, combined with information from the literature, led to the conclusion that Cu^{2+} and Fe^{3+} accelerated the denaturation of ovalbumin by promoting the browning reaction of ovalbumin with glucose, but Na^+ had no effect on it.

Solutions of copper, iron, zinc, or cadmium normally precipitate metal hydroxide under the alkaline pH value. However, no precipitate appears if a complexing agent is present which removes free metal ions from the solution. If amino acids or peptides such as L-lysine, L-glycyl-L-leucine (Nakasuka et al., 1975), and L-histidine + L-threonine (Freeman and Martin, 1969) exist together, copper is soluble even in the alkaline solutions. Tanford (1952) investigated the interaction between bovine serum albumin and a number of metals (i.e., Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}) and reported that the principal site on the protein molecule responsible for metal binding was the imidazole groups above pH 7. This might be proof that at pH 10 copper and iron had combined with ovalbumin under the existing conditions in our experiments.

Certain metal ions promote Schiff base formation by forming stable complexes and thereby providing a more favorable free-energy reaction (Leussing and Bai, 1968). However, a study of the rate of formation of *N*-salicylideneglycinate has shown that Cu^{2+} and Ni^{2+} are kinetically inactive with the highly stable Schiff base complexes being formed through proton-catalyzed paths (Bai and Leussing, 1967).

The isoelectric points of unstored samples did not show a great change, compared with the 25% loss of free amino group content in the freeze-drying process during sample preparation. This is natural from the fact that nonionized ε-amino groups reacted with carbonyl groups in the first step of the Maillard reaction. In the initial stage of the reaction, comparing the variation of free amino groups and the remaining ratios of basic amino acids with the decrease of α-helix content among OVG, OVG-Na, OVG-Cu, and OVG-Fe, it could be said that amino groups on the surface of ovalbumin were at first blocked by the added glucose before denaturation in spite of the presence or absence of metal ions in the reaction mixture. The net negative change on the protein might increase with the progress of the Maillard reaction, because the decrease of the positive charge occurs in terms of the irreversible transformation of NH_3^+ to NH_2, which can be blocked by a carbonyl group. The ways in which arginine residues participate in the reaction have not been demonstrated clearly thus far. The changes in the charge balance of the ovalbumin molecules might bring about a state in which the unfolding of proteins is encouraged. It has been demonstrated that maleylation of ε-amino groups of ovalbumin caused appreciable but incomplete unfolding of ovalbumin (Qasin and Salahuddin, 1979). This incomplete unfolding, derived from the charge imbalance, might progress further with advances of the browning reaction which was positively catalyzed by transition elements such as Fe^{3+} and Cu^{2+} and led to the increase of denaturation and insolubility of ovalbumin. These findings might be corroborated by observations revealing that traces of cupric salts had no effect on the rate of loss of amino groups of protein (Mohammad et al., 1949), metal ions catalyzed oxidative reaction of ascorbic acid (Taqui Khan and Martell, 1967) or catechols

(Tyson and Martell, 1972), and Fe^{3+} promoted the destruction of Amadori rearrangement products in the oxygen-dependent browning system of a glucose–diglycine mixture (Hashiba, 1975).

The metal-binding proteins, such as conalbumin from egg white (Azari and Feeny, 1961) and transferrin from serum (Koechlin, 1952), have a greater stability to proteolytic hydrolysis and thermal denaturation. In the stored ovalbumin–Cu^{2+} system (OV–Cu-IV), few effects on the denaturation and solubility of ovalbumin could be found even if metal bound to the ovalbumin in any form. Although copper catalyzes oxidation steps involving cystines–cysteines (Feeney et al., 1956), which might be involved in the aggregation phenomenon of denatured protein, this action might be negligible in the OV–Cu-IV system used.

The difference in the capacity to accelerate the Maillard reaction between Fe^{2+} and Fe^{3+} reflects the possibility that the first step of metal catalysis is "oxidation activation" involving the reduction of metal.

It has been clearly demonstrated that there are many species of minerals like calcium, copper, iron, magnesium, manganese, potassium, sodium, and zinc in egg white (Cotterill et al., 1977). In the present study, the effects of only three kinds of metals on the browning reaction in the ovalbumin–glucose mixture were studied. However, it is considered that Cu^{2+} and Fe^{3+} might accelerate the browning reaction in the egg white solid–glucose system reported in the previous study (Kato et al., 1978). The addition of a mere 0.003 ppm of manganese was found to inhibit the rate of browning reaction of the glucose–glycine system in air or oxygen (Bohart and Carson, 1955). Attention must be paid to the existence of trace metals in the studies on the rate of the overall browning reaction in the complex system such as egg white.

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Binding of Methylmercury to Ovalbumin as Methylmercuric Cysteine

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After the administration of methylmercury in the form of $CH_3{}^{203}HgCl$ to laying hens, the egg ovalbumin was isolated, and the binding of ^{203}Hg was determined. Enzymic hydrolysis of ovalbumin, followed by covalent chromatography of the hydrolysate on 2-pyridyl-S-S-propyl-Sepharose, enabled the separation of a ^{203}Hg-labeled digestion product. Amino acid analysis of the latter, after acid hydrolysis and performic acid oxidation, produced only cysteic acid. The hydrolyzed, but unoxidized, sample had an elution time different from that of cysteine or cystine. The ^{203}Hg-labeled, Sepharose-bound fraction showed a higher R_f value than either cysteine or cystine but a value less than that of methionine. It had a mobility similar to that of methylmercuric cysteine prepared in vitro. It was suggested that the fraction separated from egg ovalbumin contained methylmercuric cysteine and that the binding of the ^{203}Hg to the cysteine probably involved the SH group.

The interactions of mercurials with proteins usually involve the SH and S–S groups, and such interactions have been linked to the toxic effects of mercury (Hg) (Vallee and Ulmer, 1972; Webb, 1966). Despite much research during the past 20 years on sulfur–mercury interactions, the relationship between this chemical reaction and toxicological observations is yet to be explained.

Many studies on Hg-binding sites of proteins have utilized albumins as model systems. Binding of the mercuric ion (Hg^{2+}) to albumins has been demonstrated in vitro, and the reactive groups of the protein were the SH groups, as in mercaptalbumin (Hughes and Dintzis, 1964), or the COOH group, as in human serum albumin (Perkins, 1961).

The formation of methylmercuric cysteine in biological systems has been reported. A study of the accumulation of methylmercury has shown that ~95% of the muscle methylmercury was in the form of the methylmercury–cysteinyl complex (Westoo, 1966). After the injection of ^{203}Hg-labeled methylmercuric chloride ($CH_3{}^{203}HgCl$) in rats, methylmercury cysteine was found in the bile (Norseth and Clarkson, 1971).

It has been reported that, when methylmercuric chloride was administered to hens, the ovalbumin contained 97% of the egg white ^{203}Hg (Magat and Sell, 1979). The data presented in this paper deal with the chemical nature of

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