Effect of Dehydroascorbic Acid on Ovalbumin

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The effect of dehydroascorbic acid (DHA), which is an oxidized form of L-ascorbic acid (AsA), on ovalbumin (OVA) turbidity was investigated. Incubating 1% OVA in 0.2 M phosphate buffer, pH 6, containing 0.05% DHA for 20 h at 50 °C produced turbidity. Giant molecules (high molecular weight (MW) products) that could not enter the large-pore spacer gel and small molecules (low-MW products) from OVA were observed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). This electrophoretic pattern was clearly different from that of the heat or isoelectric coagulum of OVA, in which the band of the small molecule was not detected. A study of the possible participating bonds involved in the formation of giant molecules was carried out. At first, we investigated whether the formation of polymer of OVA with DHA depended on SH groups. The turbidity between SH-modified and -unmodified OVA was hardly different. Next, hydrophobic OVA, about 1.4 times more hydrophobic than nonhydrophobic OVA, and nonhydrophobic OVA were prepared, and the turbidities of both OVAs with added DHA were measured. After 20-h incubation, the absorbance at 600 nm of hydrophobic OVA was about 3 times as high as that of nonhydrophobic OVA. These results suggest that not the sulfhydryl group but the hydrophobic interaction is mainly responsible for formation of the high-MW polymer of OVA by DHA.

Since L-ascorbic acid (AsA) had an effect similar to that of an oxidizing agent in bread dough, first discovered by Jorgensen in 1939, it has been used widely as a dough improver in the baking industry. This effect was also observed in "surimi", raw fish meat paste, and AsA has been used in the "Kamaboko" (heat-induced fish gel product made from surimi) industry.

Though various mechanisms of improvement have been reported, it is generally accepted that AsA is rapidly oxidized to dehydroascorbic acid (DHA) in bread dough or surimi by oxidase or other factors and DHA as well as other oxidizing agents oxidize sulfhydryl compounds to disulfides (SH to SS) and form intermolecular bonds that increase gluten or surimi strength (Tsen and Bushuk, 1963; Tsen, 1965; Meredith, 1965; Bauernfeind and Pinkert, 1970; Yoshinaka et al., 1972; Elkassabany and Hoseney, 1980; Elkassabany et al., 1980; Nicolas et al., 1980). However, Zenter (1968) and Johnston and Mauseth (1972) speculated that the highly polymerized protein was accessible to hydrogen bond breaking by AsA on the basis of a somewhat similar effect of adding urea and other agents affecting hydrogen binding. On the other hand, Ewart (1985) proposed that the "end-blocking effect" of low molecular weight (MW) thiols (e.g., reduced glutathion or cysteine) prevented these SH groups from participating in desirable SH–SS interchange reactions and that oxidative agents were effective to prevent the end-blocking effect due to the reaction with low-MW thiols. Recently, Pfeilsticker and Marx (1986) reported that intermolecular condensation products formed with DHA and amino groups of proteins might contribute to improved baking quality.

However, the dough or surimi system is so complex that there are a number of unclarified points regarding the action of DHA against protein. Thus, ovalbumin (OVA), which has some free SH, was chosen as a protein source in this study to clarify the action of DHA against protein. Because wheat flour or muscle protein contains free sulfhydryl groups and there is possible polymerization by DHA in proteins containing the free SH group, we investigated the participation of SH group in the high-MW polymer formation of OVA, followed by examining the involvement of hydrophobic interaction.

MATERIALS AND METHODS

Materials. DHA was prepared from AsA according to the method of Kenyon and Munro (1948). OVA (A 5503) from Sigma Chemical Co. and AsA, 8-anilino-1-naphthalenesulfonic acid (ANS), and sodium dodecyl sulfate (SDS) from Nakarai Chemicals Ltd. were used. The other chemicals used were reagent grade. When OVA was applied to SDS–polyacrylamide gel electrophoresis (PAGE), subbands above the main one were observed. Because these subbands almost disappeared with addition of 2-mercaptoethanol, the OVA might have contained some dimer or trimer and a little contamination. Such OVA containing di- or trimers was used in our experiment.

Measurement of Turbidity of OVA. A mixture of 1% OVA and 0.05% DHA in 0.2 M phosphate buffer, pH 6, was incubated at 50 °C for 20 h. Turbidity was measured by the absorbance at 600 nm. As OVA turbidity after incubation depended on each lot number of commercial-grade OVA, the change in turbidity of OVA by DHA was followed whenever a commercial grade of OVA having a different lot number was obtained. To study the effects of pH on OVA turbidity, a 1% OVA solution with or without 0.05% DHA was incubated at 50 °C for 20 h in the region pH 3–10 and the absorbance at 600 nm was measured. A 0.2 M lactate buffer was used at pH 3, 0.2 M acetate buffer at pH 4 and 5, 0.2 M phosphate buffer at pH 6–8, and 0.2 M borate buffer at pH 9 and 10. A 1% OVA solution without DHA was also incubated at 60 °C for 20 h at pH 6 to produce thermal aggregation of OVA (Hegg and Löfqvist, 1974).

SDS-PAGE Analysis. After incubation at 50 °C, 1 mL of OVA solution was mixed with an equal volume (v/v) of 0.125 M Tris-HCl buffer, pH 6.8, containing 4% SDS and 20% glycerol. After being heated at 100 °C for 5 min, the mixture was applied to SDS-PAGE. Electrophoresis was carried out on slab gel at 20 mA for 3–4 h according to the method of Laemmli (1970). Some OVA samples incubated under various conditions were also used for SDS-PAGE analysis in the same manner as described above.

Preparation of SH-Modified or -Unmodified OVA. A 0.45% OVA solution in 0.05 M phosphate buffer, pH 8, containing 4 M urea, 2 M guanidine hydrochloride, and 0.01 M

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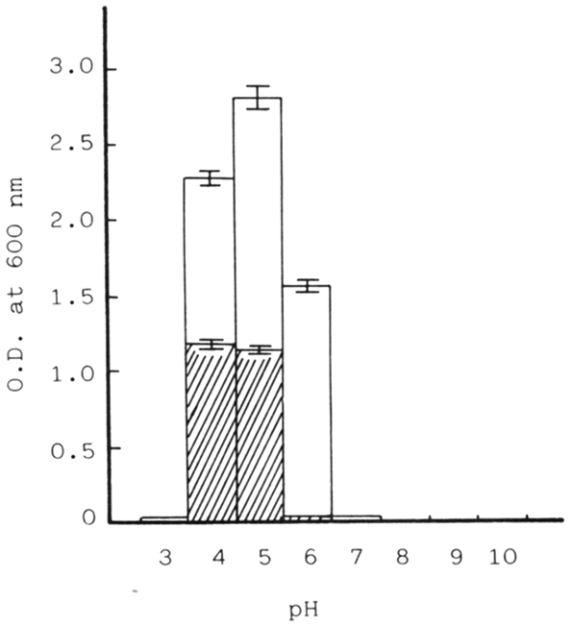

Figure 1. pH-dependent turbidity of OVA. 1% OVA solution with or without 0.05% DHA in each pH was incubated at 50 °C for 20 h, and its absorbance at 600 nm was measured. Key: shaded parts, without 0.05% DHA; open plus shaded parts, with 0.05% DHA. The bars show the standard deviations of duplicate determinations.

iodoacetamide was incubated at 25 °C for 15 min and centrifuged at 30000*g* for 10 min. Supernatant (20 mL) was applied to a column (2.5 × 4.5 cm) of Sephadex G-25 equilibrated with 0.05 M phosphate buffer, pH 8.0. Fractions having absorbances above 1.0 at 280 nm were collected and dialyzed against 0.2 M phosphate buffer, pH 6, overnight. The dialysates were centrifuged at 30000*g* for 10 min, and the supernatant was adjusted to 0.25% of protein concentration. In this OVA sample just one of three free SH groups per mole was blocked. SH-unmodified OVA was prepared by the same procedure except that 0.45% OVA was incubated in the described buffer without iodoacetamide and measured for free SH groups. The SH-unmodified OVA contained three free SH groups per mole.

Protein concentration was determined by using $E^{0.1\%}_{1cm}$ = 0.648 at 280 nm.

Measurement of SH Groups. The number of SH groups was determined by the modified method of Ellman (Ellman, 1959; Glazer et al., 1975a,b). Commercial OVA preparations had about three SH groups per mole in spite of the difference in lot numbers.

Measurement of Circular Dichroism. The circular dichroism spectra were measured in a 10-mm path length cell at the room temperature in a Jasco J-500 automatic recording spectropolarimeter. Molar ellipticity was calculated on the basis of the mean residue weight of 119.48. For the analysis of circular dichroism spectra, the method of Baker and Insenberg (1976) was used. The circular dichroism spectral value is the mean of two independent determinations.

Measurement of Surface Hydrophobicity of OVA. The change of surface hydrophobicity in 1% OVA in 0.2 M phosphate buffer, pH 6, with or without 0.05% DHA during 44-h incubation at 50 °C was measured by using SDS binding capacity represented as micrograms of SDS bound to 500 μg of protein as described by Kato et al. (1984).

Preparation of Hydrophobic or Nonhydrophobic OVA. A 0.45% OVA solution in 0.2 M phosphate buffer, pH 6, containing 4 M urea and 1.75 M guanidine hydrochloride was incubated at 25 °C for 15 min and centrifuged at 30000*g* for 10 min. A 20-mL portion was applied to a column (2.5 × 45 cm) of Sephadex G-25 equilibrated with 0.2 M phosphate buffer, pH 6. Elution was carried out with the same buffer, and 5-mL fractions were collected. The fractions having absorbance above 2.0 at 280 nm were concentrated by ultrafiltration. Nonhydrophobic OVA was prepared by the same method except with the 0.2 M phosphate buffer, pH 6, without urea and guanidine hydrochloride.

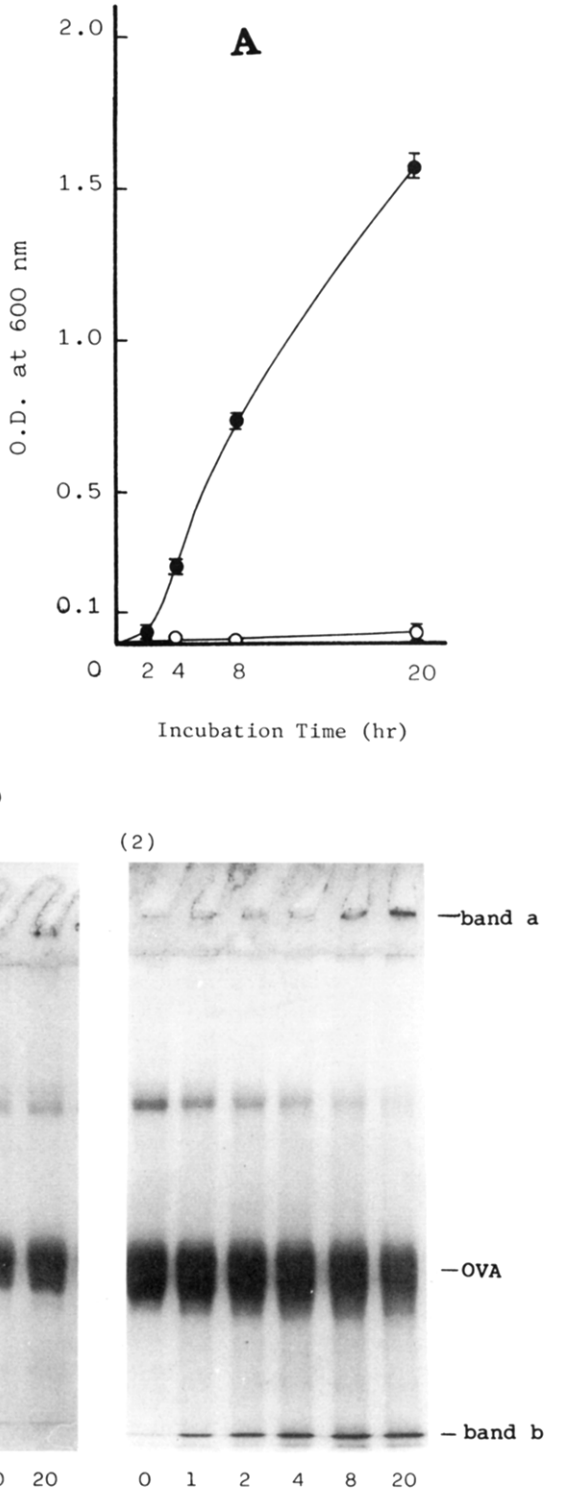

Figure 2. Changes of absorbance at 600 nm and SDS-PAGE pattern during incubation of OVA: (A) change of absorbance at 600 nm; (B) change of SDS-PAGE pattern of OVA incubated with (2) or without (1) 0.05% DHA. Conditions: 1% OVA in 0.2 M phosphate buffer, pH 6, incubated at 50 °C for 20 h. Key: (●) with 0.05% DHA; (○) without DHA. The bars show the standard deviations of duplicate determinations.

A part of the hydrophobic or nonhydrophobic OVA sample was diluted to 0.1% concentration with the same buffer, and an equal volume of 0.5 mM ANS was added. The ANS–OVA conjugates were excited at 375 nm, the relative fluorescence intensity was measured at 488 nm in a Jasco spectrophotometer, Model

FP-550, and its fluorescences intensity was compared with that of nonhydrophobic OVA. The intensity of hydrophobic OVA at 0.31 was about 1.4 times greater than that of nonhydrophobic OVA at 0.22.

Influence of the Blocked SH Group or Increasing Surface Hydrophobicity on Turbidity of OVA. The SH-modified or -unmodified OVA and hydrophobic or nonhydrophobic OVA solution containing no DHA, at pH 6, produced turbidity during 20-h incubation at 50 °C, whereas native OVA did not. As the turbidity of the 1% solution of these preparative OVAs without DHA was too high to measure with a spectrophotometer, protein concentrations of 0.25% in SH-modified or -unmodified OVA and of 0.5% in hydrophobic or nonhydrophobic OVA were used. DHA concentration was increased to 0.2% in order to spread the difference of turbidity between mixtures with and without DHA. Incubation temperature and pH were the same as described in Measurement of Turbidity of OVA. Unless otherwise noted, in all measurements, at least, duplicate determinations were independently carried out and the averages ± standard deviations were calculated.

RESULTS AND DISCUSSION

Effects of pH, Incubation Time and Temperature, and DHA on OVA Turbidity. The results from turbidity of OVA in the region pH 3–10 are shown in Figure 1. OVA solution without DHA hardly produced turbidity at pH 3 and >6, while it yielded a substantial amount of turbidity at pH 4 and 5. Hegg and Löfqvist (1974) investigated the temperature of aggregation at different pH values for a commercial grade of OVA and observed that the thermal aggregation curve versus pH for OVA dissolved in glass-distilled water was characterized by a broad pH interval from pH 5 to ≈9, in which the aggregation temperature was practically constant below 60 °C with the absolute minimum at pH 5.5. The differences in pH ranges of our experiment from those of Hegg and Löfqvist (1974) may be, in part, due to differences in solvents used. Later, Hegg et al. (1979) reported that the thermal aggregation temperature of OVA was 65 °C in the region pH 4–5.5, which is near the isoelectric point (pH 4.5) of pure OVA. Thus, the cause of turbidity at pH 4 and 5 at 50 °C (Figure 1) may be mainly due to its isoelectric point.

In the region pH 4–6 DHA accelerated turbidity of the OVA solution (Figure 1). Especially at pH 6, the turbidity of the OVA solution was solely due to the presence of DHA. Thus, to investigate the effect of DHA on OVA, the incubation was carried out at pH 6.

The changes occurring during incubation at 50 °C, pH 6, are shown in Figure 2. The turbidity of OVA without DHA was nearly undetectable irrespective of incubation time whereas that of OVA with DHA increased sharply with an increasing incubation time, showing an optical density of around 1.6 after 20 h (Figure 2A). The electrophoretic patterns are shown in Figure 2B. No significant change was observed by incubating the OVA without DHA (Figure 2B, lane 1). On the other hand, when DHA was added to OVA, band b in the lowest area appeared at the 1-h incubation and increased gradually accompanied by a decrease in OVA band intensity. Band a of a high-MW giant molecule, which could not enter the large-pore spacer gel, was produced after 8-h incubation. Thus, both high- and low-MW products were simultaneously produced from OVA in the presence of DHA. This result suggests that at first DHA produced some small peptides from OVA and then later formed some polymers. Gutteridge and Wilkins (1983) reported that proteins were damaged by •OH radicals. As oxygen radicals were produced during the autoxidation of AsA (Murata and Kitagawa, 1973; Shinohara et al., 1983; Chiou, 1983, 1984), the formation of small peptides from OVA by DHA may depend on the cut of peptide bonds by oxygen radicals during the oxidation of DHA.

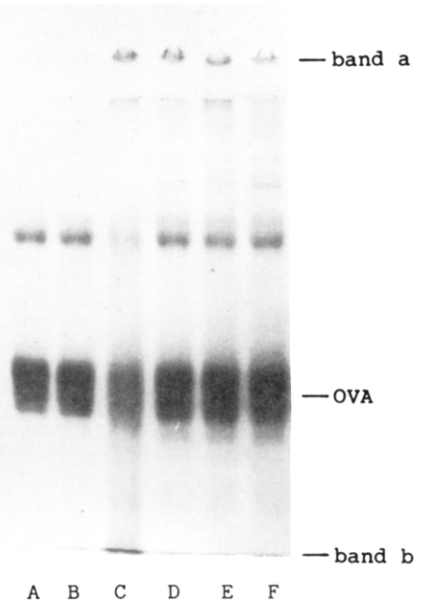

Figure 3. Difference of SDS-PAGE patterns of OVA incubated under various conditions: (A) native OVA; (B) OVA incubated without DHA at 50 °C at pH 6; (C) OVA with DHA at 50 °C at pH 6; (D) OVA without DHA at 60 °C at pH 6; (E) OVA without DHA at 50 °C at pH 4; (F) OVA without DHA at 50 °C at pH 5.

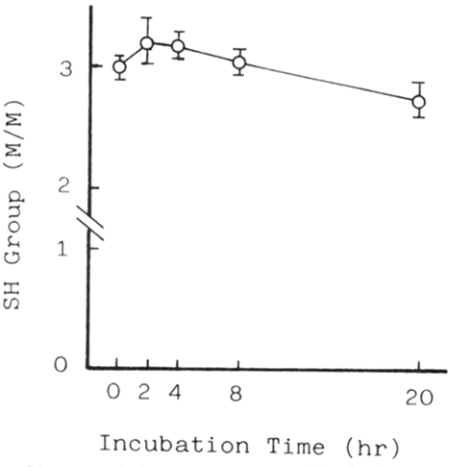

Figure 4. Change of the number of sulfhydryl groups of OVA during incubation at 50 °C, pH 6. The bars show the standard deviations of duplicate determinations.

The electrophoretic patterns of OVA samples incubated for 20 h under various conditions are shown in Figure 3. The incubated OVA sample (lane B) showed a pattern identical with that of native OVA (lane A). However, incubated OVA in the presence of DHA (lane C) produced both large-molecule band a and small-molecule band b. On the other hand, OVA solution incubated under other conditions (lanes D–F) produced high-MW band a but not low-MW band b, suggesting that the mechanism of turbidity formation of OVA solution with DHA differs from that by isoelectric techniques (lanes E and F) or heat coagulation (lane D).

Effect of SH Group Modification on OVA Turbidity. The investigation of participating bonds in the polymer formation of OVA (electrophoretic bands a, Figures 2 and 3) was carried out. At first we examined whether the formation of the high-MW polymer of OVA

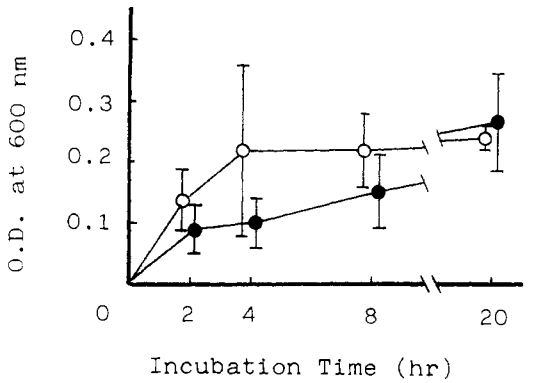

Figure 5. Influence of sulfhydryl groups on turbidity of OVA. Changes of absorbance at 600 nm: (●) sulfhydryl group modified OVA; (○) sulfhydryl group unmodified OVA. The bars show the standard deviations of four replicate determinations.

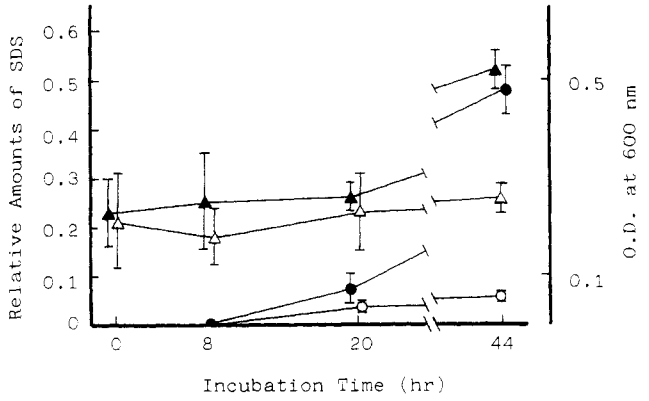

Figure 6. Change of surface hydrophobicity (micrograms of SDS bound to 500 μg of protein) of 1% OVA solution, pH 6, incubated at 50 °C: (▲) with 0.05% DHA; (△) without DHA. Change of turbidity (absorbance at 600 nm) of 1% OVA solution, pH 6, incubated at 50 °C: (●) with 0.05% DHA; (○) without DHA. The bars show the standard deviations of three replicate determinations.

Table I. Change of Conformation of OVA by Sulfhydryl Group Modification[a]

	native OVA	SH-unmodified OVA	SH-modified OVA
α-helix	18.5	17.6	18.2
anti-β-structure	19.8	22.0	24.0
parallel β-structure	4.7	5.4	6.5
random coil	38.1	36.3	33.1

[a] Sulfhydryl group unmodified or modified OVA was prepared as described in Material and Methods, and circular dichroism spectra of both OVAs were measured and compared with one of native OVA. The circular dichroism spectra value is the mean of two independent determinations.

by DHA depended on SH groups or not. The change in amount of SH groups in OVA during incubation at 50 °C, pH 6, was followed (Figure 4). No significant decrease in SH groups of OVA was observed during incubation, suggesting that SH groups were not involved in the formation of polymer. Although it is known that the number of SH group per mole of OVA is 4, the commercial grade of OVA contained approximately three SH groups per mole.

The structures of two OVA preparations (SH-modified and -unmodified) were compared with that of native OVA by circular dichroism (Table I). No significant difference was detected among the structures of each OVA preparation, suggesting that the structure of OVA hardly changed. However, when these two preparative OVA solutions, at pH 6, without DHA were incubated at 50 °C, they produced turbidity, whereas native OVA solution did not. It may be considered that hydrogen bond breaking might have occurred partly by denaturants (urea and guanidine hydrochloride) during preparation and the structure might have changed slightly even though the difference of the structure was hardly detected with circular dichroism. The participation of the SH group in the formation of polymer of OVA by DHA was investigated with these two preparative OVAs under the condition supplying the largest difference between turbidity of both preparative OVA solutions (0.25% OVA, 0.2% DHA) (Figure 5). The difference between processes of the turbidity in both OVAs was not significantly shown, indicating very little effect of modification of SH groups on OVA turbidity. This result strongly suggests that the SH group may not be responsible for the polymerization of OVA by DHA. Our results show the mechanism of polymer formation of OVA by DHA to be different from the mechanism in dough and in surimi reported by many researchers who explained that DHA oxidized sulfhydryl compounds to disulfides and protein polymers are increased by formation of intermolecular bonds (Tsen and Bushuk, 1963; Tsen, 1965; Meredith, 1965; Bauernfeind and Pinkert, 1970; Yoshinaka et al., 1972; Elkassabany and Hoseney, 1980; Elskassabany et al., 1980; Nicolas et al., 1980).

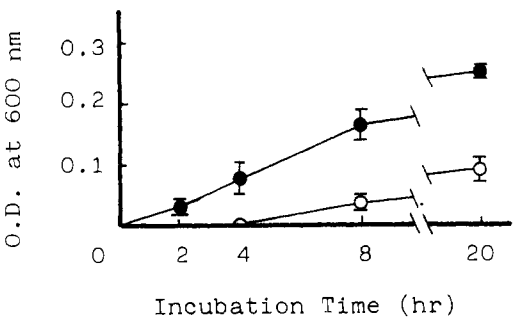

Figure 7. Effect of surface hydrophobicity on turbidity of OVA (0.5% OVA solution, pH 6, with 0.2% DHA incubated at 50 °C): (●) hydrophobic OVA; (○) nonhydrophobic OVA. The bars show the standard deviations of duplicate determinations.

Effect of Surface Hydrophobicity on OVA Turbidity. The change of surface hydrophobicity during 44-h incubation at 50 °C, pH 6, was investigated (Figure 6). The turbidity and surface hydrophobicity of the OVA solution without DHA hardly changed with incubation time. On the other hand, turbidity and surface hydrophobicity of OVA solution with DHA increased with increasing incubation times, suggesting the participation of hydrophobic interaction in the formation of polymer. At the 44-h incubation, the surface hydrophobicity was nearly 2-fold greater in the presence of DHA (Figure 6). The difference between turbidity of hydrophobic and nonhydrophobic OVA by DHA was measured (Figure 7). After 20-h incubation at 50 °C and pH 6, the absorbance at 600 nm of hydrophobic OVA was about 3 times as high as that of nonhydrophobic OVA. This shows that turbidity of OVA solution caused by DHA is related to the amount of surface hydrophobicity (Figures 6 and 7). Perhaps the cause of turbidity in the four preparative OVA solutions containing no DHA might have been due to an increase in surface hydrophobicity derived from hydrogen bond breaking by using urea and guanidine hydrochloride during preparation. The same reason might apply to the differences in turbidity depending on the lot numbers of commercial OVA.

In this study, we elucidated that not the SH group but the hydrophobic interaction is mainly responsible for formation of the high-MW polymer of OVA by DHA.

Registry No. DHA, 490-83-5; AsA, 50-81-7.

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