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Conformational Change of Hen Egg Ovalbumin during Foam Formation Detected by 5,5′-Dithiobis(2-nitrobenzoic acid)

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5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) does not react with native ovalbumin that has four cysteinyl residues at neutral pH. When ovalbumin solution was made to foam in the presence of DTNB, the liquid drained from the foam was yellow because of thionitrophenolate anion. Analysis of the drained fluid and the coagulum obtained from the foam showed that two of the cysteinyl residues in the ovalbumin molecule had reacted with DTNB. These results indicated that a conformational change in the ovalbumin molecules occurred during foam formation. Analysis of the coagulum obtained from the foam prepared without DTNB was consistent with the other results for its molecular properties and sulfhydryl level.

Functional properties of food protein have been reviewed and discussed from both fundamental and applied aspects, and foaming is one such important functional property (Halling, 1981; Cheftel et al., 1985; Stainsby, 1986). Using model systems, many workers have studied the behavior of protein at the air–water interface (MacRitchie, 1978; Graham and Phillips, 1976, 1979a–c; Tornberg, 1978a, b; Waniska and Kinsella, 1985). Surface tension and surface viscoelasticity of protein solutions have been investigated in terms of foaming properties. These physical experiments showed that proteins having a flexible or amphipathic structure (or both), such as casein or gelatin, change conformation at the air–water interface. Some globular proteins seem to unfold at this interface by orienting themselves with their hydrophobic area toward the air to decrease the surface energy of the system. Conformational change or denaturation of globular protein also seems likely because some enzymes are inactivated by foaming (Lee and Hairston, 1971; Donaldson et al., 1980; Shibata and Doi, 1984) and because the surface tension decay of a globular protein solution continues for a long time (Kitabatake and Doi, 1982). However, direct evidence of the conformational change of globular proteins at the air–water interface or by foaming has not been reported. Here, we report on the conformational change of ovalbumin molecules that gives rise to foam formation by a change in the reactivity of the sulfhydryl groups.

MATERIALS AND METHODS

Chemicals. The 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), *N*-ethylmaleimide (NEM), iodoacetamide (IAAmide), guanidine hydrochloride (GuHCl), and urea obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan), were special grades for sulfhydryl group assays. Other chemicals were reagent grade and were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), or Nakarai Chemicals, Ltd.

Two kinds of buffer were used. Buffer A was 20 mM sodium phosphate buffer, pH 7.5, containing 0.1 mM EDTA, and buffer B was 40 mM sodium phosphate buffer, pH 8.0, containing 0.48% SDS, 8 M urea, and 1 mM EDTA.

Preparation and Assay of Ovalbumin. The ovalbumin was purified from fresh egg white by the procedure of Soerensen (1915–1917) with minor modifications. The distilled water used, which contained EDTA (0.1 mM), was bubbled beforehand with N_2 gas to remove dissolved oxygen. The ovalbumin was crystallized a total of five times. The ovalbumin crystal obtained was stored as a suspension at 4 °C with 0.03% NaN_3 and 0.1 mM EDTA. Before being used, the crystal was collected by centrifugation and either dialyzed against buffer A or gel filtrated with Sephadex G-50 with the same buffer. The desalted ovalbumin sample was used for the foaming experiments. The purified ovalbumin preparation showed a single band on SDS–polyacrylamide gel electrophoresis. Ovalbumin concentrations were calculated from the absorbance at 280 nm based on the value of $E_{1\mathrm{cm}}^{1\%}$ = 7.12 (Glazer et al., 1963).

Foaming of Ovalbumin Solution. First, 15 mL of ovalbumin solution (4% w/v) dissolved in buffer A was placed in a 50-mL cylindrical glass vessel (26-mm i.d. × 93-mm depth). The ovalbumin solution was stirred for 1 min at 10000 rpm in a homogenizer (Ace type, Nihon Seiki Kaisha Co., Ltd.) equipped with a holder filled with water of a constant temperature (25 °C) and a rotating six-bladed knife. After the stirring, the knife was withdrawn and the glass vessel containing the ovalbumin foam was tightly sealed and left for 1 h at 25 °C. The drained liquid (Drain 1 in Figure 1) was separated from the foam by decantation, and the foam was collapsed by centrifugation at 3000*g* for 10 min at 25 °C. The supernatant (Drain 2) was removed by decantation. Then, 50 mL of buffer A was added to the precipitate and the mixture centrifuged again in the same way. The precipitate obtained was transferred to a glass filter (Japanese Industrial Standard 3G3; pore size 20–30 μm) and washed with 100 mL of buffer A with aspirating. The remaining precipitate was used in the experiment as coagulum. The steps are shown in Figure 1.

Measurement of Reduced Viscosity. Viscosity was measured on the Ostwald capillary viscometer, with an efflux time of 140 s for water at 25.0 ± 0.2 °C. NaCl was added to all samples at the concentration of 0.1 M. The sample was diluted with buffer A.

Measurement of Sulfhydryl Levels. To 0.1 mL of ovalbumin solution (4% w/v) was added 4.9 mL of buffer B. Then, 33 μL of 10 mM DTNB solution (39.6 mg of DTNB/10 mL of 40 mM sodium phosphate buffer, pH 7.0) was added to the mixture and the whole incubated for 1 h at 50 °C. After the mixture was cooled, the absorbance at 412 nm was measured. The reference was distilled water instead of ovalbumin solution. The molar extinction coefficient of 13600 M^{-1} cm^{-1} at the sulfhydryl groups in the ovalbumin molecule modified by DTNB was used (Ellman, 1959). The value of the sample is against that of the reference.

To measure the sulfhydryl in ovalbumin in the coagulum fraction, 5.0 mL of buffer B bubbled with N_2 was added to about 0.1 g of the coagulum, and the tube containing the sample was tightly sealed. The sample was incubated for 3 h with shaking at 50 °C to dissolve the coagulum.

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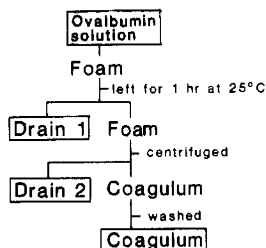

Figure 1. Fractionation of foam. See Materials and Methods for details.

Then the absorbance at 280 nm was measured to find the protein concentration. After this, 33 μL of the 10 mM DTNB solution described above was added and the resultant mixture incubated for 1 h at 50 °C. The sample was measured for its absorbance at 412 nm. $E_{1cm}^{1\%}$ at 280 nm of the dissolved coagulum was taken to be 7.12. The sulfhydryl content was calculated by moles of sulfhydryl per mole of ovalbumin.

Assay of the Disulfide Level. First, 2.0 mL of buffer B containing 10 mM DTT was added to 0.5 mL of ovalbumin solution, which was bubbled with N_2 gas to reduce disulfide bridges, and the resultant mixture incubated for 1 h at 50 °C. Then, 0.5 mL of the incubated sample was put on a Sephadex G-25 column (1.2-cm i.d. × 30-cm height) equilibrated with buffer B. Buffer B was bubbled with N_2 gas just before use to remove dissolved oxygen. Elution was done using buffer B at the rate of 1.0 mL/min. Fractions of 1 mL of the eluate were collected, and DTNB solution was immediately added to a portion of the fraction to measure the sulfhydryl level, calculated as described above. The absorbance at 280 nm of each fraction was measured. DTT also absorbes at 280 nm, so the elution pattern of both protein and DTT could be followed by measurement of absorbance at 280 nm. DTT was completely separated from the protein fractions by this chromatography. When the coagulum was reduced by this method, sample solubilized with buffer was used as the starting material as described in the preceding section.

Foaming of Ovalbumin Solution in the Presence of DTNB and Measurement of Ovalbumin Modified with DTNB by Foaming. To 15 mL of ovalbumin solution (4% w/v) in buffer A was added 59.0 mg of DTNB, and foam was produced with stirring as above. The foam was incubated at 25 °C for 1 h, and fractions of Drains 1 and 2 of the coagulum were prepared as shown in Figure 1. As a control experiment, an ovalbumin solution without foam was incubated with DTNB under the same conditions. The absorbance at 412 nm of the samples was measured. For the reference solution, 59.0 mg of DTNB in 15 mL of buffer A was also stirred, incubated, and measured for absorbance at 412 nm. This value was subtracted from the values of the above samples.

Then, 0.5 mL of the original ovalbumin solution incubated with DTNB or Drain 1 or 2 was put on the Sephadex G-25 column (Pharmacia; prepared, disposable column PD-10), equilibrated with buffer A. Each milliliter was fractionated, and the absorbance at 280 and 412 nm was measured. To 0.5 mL of each fraction was added 0.02 μL of 2-ME, and the absorbance at 280 and 412 nm measured again. From these results, the amount of TNB-modified ovalbumin was calculated as moles per mole of ovalbumin.

The coagulum obtained was washed with 40 mL of buffer A on a glass filter with aspiration. The washed coagulum was dissolved in buffer B and its absorbance at 280 and 412 nm measured. Then, 2-ME was added to the solubilized coagulum and the absorbance at 280 and 412 nm read again. The same experiment using the solution without ovalbumin was done as a reference.

Table I. Sulfhydryl Contents of Ovalbumin in Drains and Coagulum

	SH found,[a] mol/mol		
fractions	without 2-ME	with 2-ME	protein rec[b]
orig ovalbumin	4.08	5.96	
Drain 1	4.14	5.56	94.5
Drain 2	4.12	5.68	3.2
coagulum	1.32	5.72	2.3

[a] Sulfhydryl content was assayed after denaturation with 0.5% SDS and 8 M urea as described in Materials and Methods. [b] After foaming, Drain 1, Drain 2, and coagulum were separated as described in the text.

Polyacrylamide Gel Electrophoresis in SDS (SDS-PAGE). To 20 μL of a 4% (w/v) ovalbumin solution (the original solution, or Drain 1 or 2, or coagulum solubilized with buffer B as described above) were added 30 μL of 50 mM IAAmide or NEM solution and 150 μL of buffer A, and the resultant mixture was incubated for 1 h at 50 °C to unfold the protein and to modify the free sulfhydryl groups. Then, 10 μL of each sample was put on a 5% polyacrylamide gel. When the coagulum was reduced by 2-ME, 5 μL of 2-ME solution was added to the 20 μL of the reaction mixture and the mixture incubated for 1 h at 50 °C. Electrophoresis in gels containing SDS was performed in a slab gel apparatus as described by Laemmli (1970). Gels were stained in a solution containing final concentrations of 0.1% Coomassie blue, 50% methanol, and 10% acetic acid and destained by diffusion in a solution of 5% methanol and 10% acetic acid.

Gel Permeation Chromatography. Ovalbumin solution, Drain 1, Drain 2, or the solubilized coagulum was added to an equal volume of 1 M Tris-HCl buffer, pH 8.5, containing 6.0 M GuHCl and 1 mM EDTA. N_2 gas was bubbled through the sample, which was then incubated for 30 min at 50 °C. DTT was added at a level 50-fold that of the sulfhydryl level in the sample, which was then incubated for 2 h at room temperature. When the sample did not need reduction, this treatment was omitted and we proceeded to the next step. Then IAAmide of double the molar quantity of DTT was added and the pH was adjusted to pH 8.0–8.5 with 0.5 M NaOH. This solution was incubated for 1 h, and 0.5 mL of the sample was put on a Toyopearl HW-60S gel (Toyo Soda Manufacturing Co., Ltd., Tokyo) column (1.5-cm i.d. × 100-cm height). Elution was done with 1 M Tris-HCl buffer, pH 8.5, containing 6.5 M GuHCl buffer and 1 mM EDTA at a flow rate of 17 mL/h, and every 0.5-mL a fraction was collected. The effluent was monitored by absorbance at 280 nm.

RESULTS AND DISCUSSION

The drainage of ovalbumin solution from foam that had been made by stirring with a homogenizer proceeded with time but was almost complete after 1 h at room temperature. The recovery of ovalbumin in the drain was 94.5% (Figure 1, Drain 1). The air bubbles coalesced, forming larger bubbles, with time; however, the film of the bubbles was elastic and stable after allowing the foam to stand for a given time. Centrifugation collapsed the foam, and Drain 2 and the coagulum fraction were obtained. As shown in Table I, most of the ovalbumin was recovered in Drain 1 and a very little in Drain 2. The protein concentrations of Drains 1 and 2 were almost the same as that of the original ovalbumin solution in spite of the formation of coagulum.

Viscosity. To find whether there is a conformational difference between the original solution and Drain 1 fraction of ovalbumin, we measured the reduced viscosities

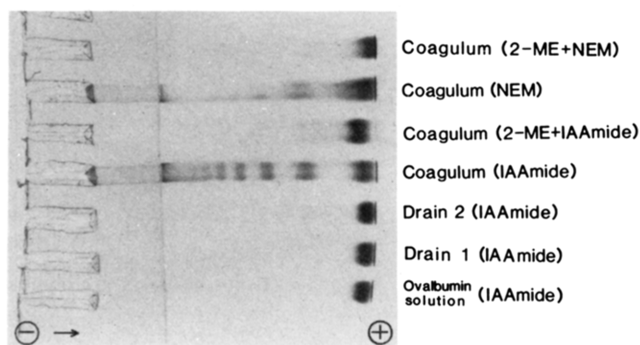

Figure 2. Polyacrylamide gel electrophoresis in SDS. Bands moved from left to right in the figure. First, 40 μg of ovalbumin in the original solution, Drain 1, and Drain 2 (in Figure 1) was applied; 80 μg of the coagulum solubilized by buffer B was then applied to the gel. Each sample was treated with IAAmide, NEM, 2-ME, or some combination as shown in the brackets before electrophoresis. Details are described in Materials and Methods.

of both solutions at each concentration and calculated the intrinsic viscosity by extrapolation of the plot. The values obtained were 4.39 and 4.45 dL/g in the original ovalbumin solution and Drain 1, respectively. The difference between them was insignificant, showing that the ovalbumin in Drain 1 was almost the same as that in the original solution in conformation.

Change in Sulfhydryl Levels. The number of sulfhydryl groups in the original ovalbumin, Drain 1, Drain 2, and coagulum, which were or were not treated with the reducing agent, was measured in the presence of urea and SDS (Table I). Differences in the original ovalbumin, Drain 1, and Drain 2 were insignificant; values were about 4 mol of sulfhydryl group/mol of ovalbumin. This value is consistent with the value of the native ovalbumin reported (Nisbet et al., 1981); however, the sulfhydryl value of ovalbumin in the coagulum was 1.32 mol/mol of ovalbumin.

After reduction of each sample by the addition of dithiothreitol, the total sulfhydryl values were about 6 mol/mol of ovalbumin, which was consistent with the reported value (Nisbet et al., 1981). These results indicate that some of the sulfhydryl residues of ovalbumin molecules in the coagulum are oxidized to form a disulfide bridge between the same molecule or different molecules during foaming. Whether this disulfide bridge forms intra- or intermolecularly was next examined.

SDS-PAGE of Fractions. SDS-PAGE of the original, Drain 1, and Drain 2 ovalbumins with sulfhydryl residues blocked by an excess of IAAmide was done without a sulfhydryl reducing agent. Figure 2 shows the single band found for each of the three samples. Electrophoresis of the coagulum under the same conditions gave many bands of higher molecular weight, including an extremely large protein that could not penetrate into the separating or stacking gel. When the coagulum was treated with 2-ME, the bands of higher molecular weight completely disappeared; there was a single band at a position close to that of the original ovalbumin. When NEM was used instead of IAAmide, the results were similar. These findings show that the reduction of sulfhydryl groups represented the oxidation of cysteines and new formation of disulfide bridges between molecules.

Gel Permeation Chromatography. To check these results, we used gel permeation chromatography of each fraction. Original, Drain 1, and Drain 2 ovalbumins modified with IAAmide centered on fraction 158 by chromatography in GuHCl with or without 2-ME (Figure 3). Coagulum solubilized by 0.5 M GuHCl in the presence

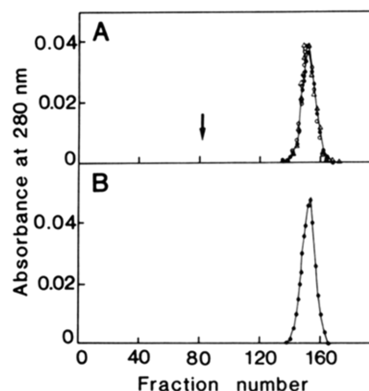

Figure 3. Elution patterns of ovalbumin in the original solution, Drain 1, and Drain 2 by gel permeation chromatography using Toyopearl HW 60-S in 6.5 M GuHCl. A: original solution (●), Drain 1 (○), and Drain 2 (△) treated with IAAmide before application on the column. B: original solution treated with both 2-ME and IAAmide before application on the column. The position of blue dextran is indicated with an arrow. Details are described in Materials and Methods.

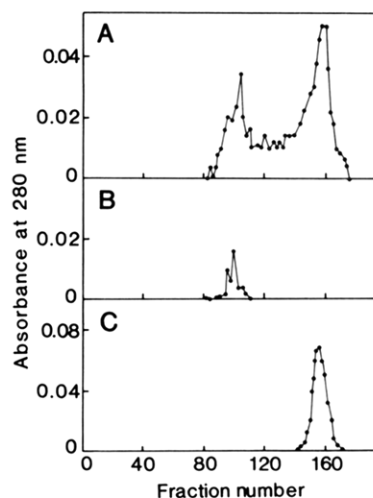

Figure 4. Elution patterns of the coagulum fraction solubilized with buffer B (see text) by gel permeation chromatography using Toyopearl HW 60 S in GuHCl; A, coagulum treated with IAAmide; B, rechromatography of 0.5 mL of the solution combined the fractions No. 103–108 in Figure 5A; C, coagulum treated with both 2-ME and IAAmide.

of IAAmide gave many peaks (Figure 4A), indicating the formation of polymers, as mentioned above. The coagulum contained some monomeric ovalbumin molecules. Blue dextran was eluted at No. 80. The polymers of the ovalbumin molecule linked together through disulfide bridges had a wide variety of molecular weights. The fractions from No. 103 to 108 were collected, combined, and rechromatographed (Figure 4B). The combined sample was recovered at a position close to that in the first chromatography, indicating that the fraction of high molecular weight seen then was not artificially formed during chromatography but was covalently formed. The coagulum was treated successively with 2-ME and IAAmide as in Figure 2 and put on the column for gel filtration. A single peak with the same elution position as that of the original, Drain 1, and Drain 2 ovalbumins seen in Figure 3 was obtained (Figure 4C). This means that the polymer in the coagulum was reduced to monomers by 2-ME and confirms that the polymer was made with disulfide bridges.

Changes in Reactivity of Cysteinyl Residues after Foaming. The results described above suggested that the conformational change in ovalbumin molecules occurred

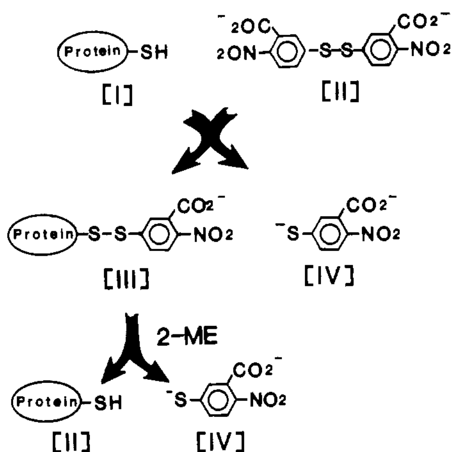

Figure 5. Reaction of sulfhydryl residues of protein with DTNB [5,5'-dithiobis(2-nitrobezoic acid)] and color development with the release of thionitrobenzoic acid (TNB). Details are described in the text.

Table II. Color Development by Foaming of Ovalbumin Solution in the Presence of DTNB

fraction	color developed by TNB (*A* 412)	SH found,[b] mol/mol without 2-ME	SH found,[b] mol/mol with 2-ME
ovalbumin soln[a]	0.000	0.00	0.02
Drain 1	0.186	0.00	0.02
Drain 2	0.236	0.00	0.03
coagulum		0.29	2.15

[a] Ovalbumin solution was kept for 1 h with DTNB without foaming. [b] Sulfhydryl content was assayed without addition of SDS and urea. Drain 1 and Drain 2 were treated with Sephadex G-25 as described in Materials and Methods before the assay. Coagulum was washed several times with buffer A and then solubilized with buffer B before the assay.

during foaming and that some of the sulfhydryl residues became reactive and formed intramolecular disulfide bridges. Direct evidence of a change in the reactivity of sulfhydryl residues was looked for by using DTNB. An ovalbumin molecule has four cysteinyl residues. These sulfhydryl groups do not react with DTNB in the native state but do react after denaturation by urea, SDS, or GuHCl. Figure 5 depicts the reaction between cysteinyl residues in the denatured protein molecule and DTNB. A cysteinyl residue in the ovalbumin molecule [I] reacts with DTNB [II], yielding a complex [III] and TNB (thionitrobenzoic acid) [IV]. Compound IV has intense absorption at 412 nm; it is yellow. Compound III has little absorbance at 412 nm, but if a reducing agent like 2-ME is added to it, it decomposes to protein and IV; this is accompanied with the new development of yellow arising from TNB [IV].

Foam was prepared from ovalbumin solution in the presence of DTNB, and Drains 1 and 2 and coagulum fractions were obtained as before. When original ovalbumin was incubated with DTNB at 25 °C for 1 h, color did not appear (Table II). However, when foam was left for 1 h, color developed, as seen by the increase in the absorbance at 412 nm of Drains 1 and 2 (Table II). This means that during formation or standing of foam cysteinyl residues, which are not reactive in the native state, were exposed to react with DTNB and that the TNB released from DTNB flowed out into the drain fraction. This means that the conformational change in the ovalbumin molecule did occur during or after foaming. The absorbance at 412 nm of Drain 2 was higher than that of Drain 1. Ovalbumin molecules modified with DTNB: [III] should be retained inside of the thin film forming a bubble.

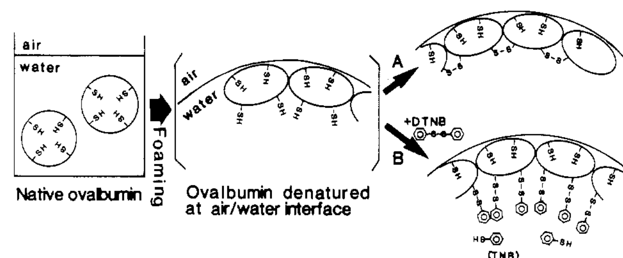

Figure 6. Proposed scheme of surface denaturation of ovalbumin by foaming (see text for details).

If it is, DTNB-modified ovalbumin should be recovered in the coagulum. To check this, the coagulum obtained was washed exhaustively with buffer A and solubilized with buffer B. Then, sulfhydryl content was assayed with and without addition of 2-ME. New color development was observed during solubilization, which corresponds to a ratio of 412 to 280 nm (0.789) corresponding to 2.15 mol of sulfhydryl groups/mol of ovalbumin already found. This means that 2.15 mol of cysteinyl residues in ovalbumin was exposed and reacted with DTNB. This value might be the same as for the sulfhydryl level reported in Table I if all of the exposed sulfhydryl residues formed a disulfide bond or reacted with DTNB. In Table I, the number of sulfhydryl groups exposed to form disulfide bridges was 2.40 mol/mol of ovalbumin, while 1.86 mol/mol of ovalbumin reacted with the DTNB by foam formation, as shown in Table II. There is a discrepancy between the numbers. However, if there is some oxidation of sulfhydryl groups during solubilization of the coagulum, or if the reactivities of the sulfhydryl groups exposed to form disulfide bridges and to form the DTNB-modified ovalbumin are different, the discrepancy is understandable. Color did not develop after the addition of 2-ME to original ovalbumin, Drain 1, or Drain 2 which had been treated by Sephadex G-25 to remove the free TNB as described in Materials and Methods. This shows that complex III was formed only in the coagulum, where the reaction of DTNB with ovalbumin molecules occurred at the air–water interface.

These results suggested that the increase in absorbance at 412 nm observed in Drains 1 and 2 (first column in Table II) arose from the conformational change in ovalbumin molecules that occurred while foam was left. Cysteinyl residues of ovalbumin were activated to react with DTNB, and the TNB produced by cleavage of DTNB flowed out of the foam into the drain.

The foam prepared from an ovalbumin solution is more stable than that from casein or gelatin solution (in preparation). Foam from an ovalbumin solution gels on the surface after being left for a certain period, which suggests that the ovalbumin molecules were adsorbed at the air–water interface and interacted with each other at the interface. In foaming of egg white, globulin plays an important role (Johnson and Zabik, 1981a); however, it seems that ovalbumin also contributes to the formation of the surface gel at the air–water interface to give a stable foam, because egg white is abundant in ovalbumin molecules, and it is reported that ovalbumin interacted with other protein components in foaming of egg white (Johnson and Zabik, 1981b).

Gel on the surface of the foam could be recovered as coagulum from the solution part of the foam. In fact, some of the cysteinyl residues of ovalbumin in the coagulum were reduced, and polymers linked by disulfide bridges were found in this fraction. This change should be accompanied by a conformational change of the molecule at the air–water interface. The process may be explained as follows. As shown in Figure 6, when an ovalbumin solution

is whipped, the ovalbumin molecules are adsorbed at the air–water interface, arrange themselves, and change their conformation to orient their hydrophobic portion in the direction of the air. This change exposes the cysteinyl residues that were buried inside of the molecule to reaction with DTNB (Figure 6A). TNB is released from the DTNB, and it develops a yellow color and is recovered in the drain fraction. On the other hand, in the absence of DTNB, the exposed cysteinyl residues might be oxidized to make disulfide bridges with neighboring cysteinyl residues of an ovalbumin molecule beside it at the air–water interface. This reaction proceeds to form polymers. Disulfide interchange reaction or intramolecular disulfide formation also might take place. The polymer formed at the air–water interface seems to produce a surface gel network, and this might be related to the stability of ovalbumin foam. However, polymer formed by disulfide bridges does not seem to be essential for stabilization of the foam, because foam prepared with ovalbumin solution in the presence of DTNB was stable for 1 h. Addition of IAAmide or NEM affected foam stability little. therefore, the formation of polymer by disulfide bridges is not the essential factor in formation of a stable ovalbumin foam. The polymer is the result of gel network formation where each denatured ovalbumin molecule interacts with others by noncovalent forces and where sulfhydryl groups are oxidized, connecting neighboring molecules. This means that the conformational change at the air–water interface strengthens the interaction between neighboring molecules by noncovalent bonding and disulfide bridges, stabilizing the foam. A surface gel network on the foam was observed by transmission electron microscopy and confirmed by surface viscosity measurements (in preparation).

A change in the reactivity of the cysteinyl residues was found in other circumstances, such as in thermal denaturation. This technique with DTNB can be used to examine fine conformational changes of protein molecules and to monitor the denaturation of proteins.

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Registry No. DTNB, 69-78-3.

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