

Inhibition against heat coagulation of ovotransferrin by ovalbumin in relation to its molecular structure

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Abstract

The effects of ovalbumin (OA) on heat coagulation of ovotransferrin (OT) were studied by heating at 65 °C for 10 min in 10 mM phosphate buffer, pH 7.0. The heat interactions between OA and OT were investigated by turbidity development and polyacrylamide gel electrophoresis. From the change in turbidities of various mixtures of 0.05% OT and OA induced by heating, it was found that OA had the inhibiting capacity against OT coagulation. The inhibiting capacity of OA was reduced by adding NaCl to OA–OT mixture on heating. The heat-denatured OA that was preheated above 70 °C exhibited markedly the inhibiting capacity against OT coagulation at 65 °C. A possible mechanism by which OA inhibited the OT coagulation was discussed. It was presumed that OA interacted mutually with OT by the electrostatic attractive force, unfolded together by heating, and then formed soluble aggregates with OT through intermolecular forces such as hydrophobic and disulfide bonds and, consequently, such a process might inhibit OT–OT interaction that led to coagulation. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Heat coagulability of egg white is one of its important functional properties; therefore, the heat denaturation and aggregation of egg white and its component proteins have been studied by many investigators. In order to elucidate the heat coagulation of egg white, heat-induced interactions among the heterogeneous proteins in egg white have been also investigated. These studies have shown that, by heat treatment, lysozyme makes insoluble aggregates with other egg white proteins, including ovalbumin (OA, Cunningham, & Lineweaver, 1967; Matsudomi, Yamamura, & Kobayashi, 1986), ovomucoid (Matsuda, Watanabe, & Sato, 1982), macroglobulin (Nakamura & Matsuda, 1983) and ovotransferrin (OT, Matsudomi, Takasaki, & Kobayashi, 1991). OT is the most heat-labile protein in the egg white and heating of egg white even at a lower temperature near 60 °C caused the coagulation of OT (Matsuda, Watanabe, & Sato, 1981; Yamashita, Ishibashi, Hong, & Hirose, 1998). Such a high heat sensitivity of OT might be

driving forces of heat coagulability of egg white. On the other hand, the heat denaturability of OT would restrict the conditions of heat pasteurization of egg white for elimination of microorganisms and the applications of egg white to heat-processed foods.

During further experiments on the heat-induced interaction of egg white proteins, we have found that OA inhibits the heat coagulation of OT on heating at 65 °C. The preparation of egg white without coagula production at temperature near 65 °C (the industrial pasteurization temperature of egg white) would be favorable to further applications of the protein as functional food materials. In this paper, we examine the factors contributing to the inhibiting capacity of OA on heat coagulation of OT, and then discuss a possible mechanism by which OA inhibits the OT coagulation on the molecular basis of OA.

2. Materials and methods

2.1. Materials

OA was isolated from fresh egg white by a crystallization method in sodium sulfate according to the

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method of Kekwich and Cannan (1936), and then freeze-dried after adjusting pH to 7.0. OT (Type I, from chicken egg white that was substantially iron-free) and *cis*-parinaric acid were purchased from Sigma Chemicals Co (St. Louis, MO). Protein solution was solubilized in a 10 mM sodium phosphate buffer (pH 7.0, heating buffer) unless otherwise mentioned. All chemicals used in this study were of reagent grade.

2.2. Heat treatment and measurement of turbidity

Protein solution (2 ml of 0.05% OT in a 10 mM sodium phosphate buffer, pH 7.0; heating buffer) was heated at a given temperature for 10 min in the sealed glass vials (Pyrex culture tubes, Corning 9826). After heating, the vials were removed and cooled immediately in an ice bath. The turbidity of solution in each vial was measured at 500 nm with a Hitachi spectrophotometer (model U-2000, Tokyo). Each vial was shaken well before measuring the turbidity to ensure a uniform suspension. The turbidity development was used as a measure of the extent of coagulation (insoluble aggregates production) of protein.

2.3. Solubilization of OT coagula

The heated OT solution was separated into a supernatant and precipitate by centrifugation at 2000×*g* for 20 min. The precipitate was washed twice with the heating buffer in order to prevent the contamination with non-precipitated proteins. The solubilization of protein in the precipitate was conducted using various agents which differ from each other according to their ability to cleave intermolecular bonds: electrostatic and hydrogen bonds (0.6 M NaCl), hydrogen bonds (1.5 M urea), hydrophobic bonds (0.5% SDS), and disulfide bonds (10 mM dithiothreitol, DTT) (Careche, Alvares, & Tejada, 1995; Kauzmann, 1959; Wall & Huebner, 1981). The precipitate was treated independently with 0.6 M NaCl (solution A), 0.6 M NaCl plus 1.5 M urea (solution B), 0.6 M NaCl plus 0.5% SDS (solution C), and 0.6 M NaCl and 0.5% SDS plus 10 mM DTT (solution D). The precipitate containing each solution was homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) at 2000 rpm for 1 min below 25 °C and then centrifuged. The supernatant fractions were analyzed by electrophoresis.

2.4. Inhibition of OA against heat coagulation of OT

The inhibition of OA against the heat coagulation of OT (0.05%) in the heating buffer was determined under heating of 65 °C for 10 min, for comparison with heat coagulation in a single system of OA or OT. After heating of the mixed solution of OA and OT, the turbidity was measured as described above. The effect of

NaCl on the inhibiting capacity of OA against the OT coagulation was also examined. The inhibiting effects of OA on the OT coagulation in the heating buffer with various NaCl concentrations (0–100 mM) were determined by measuring the turbidity after heat treatment described above.

2.5. Gel electrophoresis

The heated protein solution was separated into a supernatant and precipitate by centrifugation at 2000×*g* for 20 min. The supernatant and precipitate fractions were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The electrophoresis was done in both reducing and non-reducing conditions in the presence and absence of 2-mercaptoethanol (2-ME), respectively. In order to assess structural changes of protein and protein interaction, native PAGE without SDS and reducing agent was also performed in 7.5% polyacrylamide gel sheet, as described by Davis (1964). In addition, bi-dimensional gel electrophoresis (native→SDS and SDS + 2-ME) was performed as follows: the first dimension was performed in native PAGE as indicated above. After the run, the first-dimension gel portion was treated with 10 vol. of treatment buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 20% sucrose) or the treatment buffer containing 0.2 M 2-ME for 30 min at 55 °C, with the solution being changed at least two times. The second dimension was performed in a 10% SDS polyacrylamide gel. All gels were fixed and stained with Coomassie Brilliant Blue Stain. Molecular weight protein standards (phosphorylase, 97 kDa; serum albumin, 66 kDa; ovalbumin, 42 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; Daiichi Pure Chemicals, Tokyo) were applied on each SDS gel for comparison.

2.6. Measurement of surface hydrophobicity

Surface hydrophobicity was measured by the fluorometric method using *cis*-parinaric acid as described by Kato and Nakai (1980). Ethanolic solution of *cis*-parinaric acid (3.6×10^{-3} M) was prepared and equimolar butylated hydroxytoluene was added as an antioxidant. Ten µl of the *cis*-parinaric acid solution was added to 2 ml of protein solutions (0.1, 0.05, 0.025, and 0.0125%). The mixtures were excited at 325 nm, and the relative fluorescence intensities were measured at 420 nm in a spectrophotofluorometer (Aminco-Bowman, J-8962). The reading of relative fluorescence intensity was adjusted to 1.0 when 10 µl of *cis*-parinaric acid solution was added to 2 ml of the heating buffer, in the absence of protein. The initial slope (S_0), fluorescence intensity/% protein, was calculated from the fluorescence intensity vs. protein concentration.

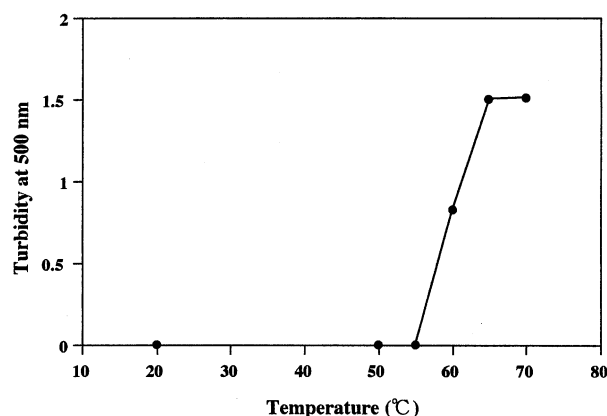


Fig. 1. Temperature dependence of the turbidity development of ovotransferrin (OT). The OT solution (0.05%, in 10 mM sodium phosphate buffer, pH 7.0) was heated for 10 min at various temperatures, and the turbidity was measured by reading absorbance at 500 nm.

2.7. Measurement of sulfhydryl (SH) groups

Free SH groups were determined using 5,5'-dithiobis (2-nitrobenzoic acid; DTNB) according to the procedure of Ellman (1959). To 1 ml of 0.1% protein solution was added 1 ml of 0.1 M Tris–glycine buffer (pH 8.0) containing 0.01 M EDTA. After incubation at 40 °C for 30 min, 50 µl of DTNB solution (4 mg in 1 ml of 0.1 M Tris–glycine buffer, pH 8.0) was added and then incubated at 25 °C for 10 min. The color was read at 412 nm. The molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ at SH groups in the OA molecule modified by DTNB was used. The SH content was calculated by moles of SH per mole of OA.

3. Results and discussion

3.1. Coagulation of OT by heating

First of all, the heat denaturation of OT was investigated. We have already reported that OT solution produced turbidity over the pH range 6.5–8.5 upon heating at 65 °C, and the maximum turbidity was observed at about pH 7 (Matsudomi, Takasaki, & Kobayashi, 1991). Then, OT solution (0.05%) in 10 mM phosphate buffer (pH 7.0, the heating buffer) was heated at temperatures from 20 to 70 °C for 10 min. Fig. 1 shows the turbidity development at each temperature plotted against the heating temperature. The OT solution did not show any change in turbidity until heating up to 55 °C, but became turbid when heated at 60 °C. The turbidity reached saturation value by heating at 65 °C, most of OT in the solution became insoluble. Precipitation appeared to be caused by aggregation of OT molecules after heat denaturation.

To elucidate the major forces contributing to the precipitate formation, the supernatant and precipitate

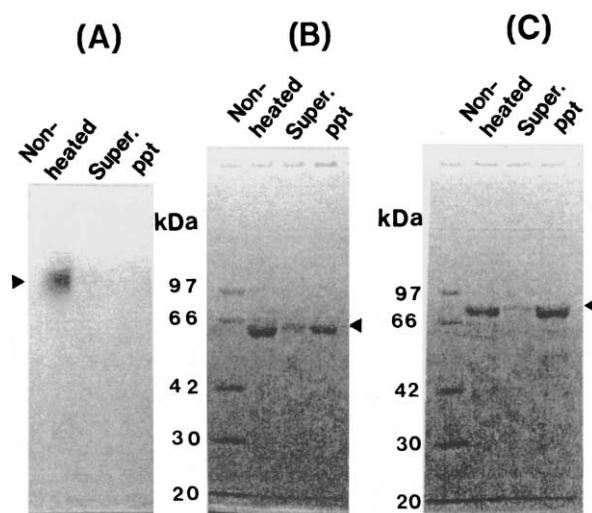


Fig. 2. Native PAGE and SDS-PAGE analysis of heated ovotransferrin (OT). A position of unheated OT is indicated by an arrow. The numbers in the figure indicate the molecular mass of marker proteins (phosphorylase, 97 kDa; bovine serum albumin, 66 kDa; aldolase, 42 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa). (A), native PAGE; (B), SDS-PAGE under non-reducing conditions; (C), SDS-PAGE under reducing conditions.

fractions of OT solution heated at 65 °C for 10 min were analyzed by native PAGE, and SDS-PAGE in the presence and absence of 2-ME. The supernatant fraction gave a single band corresponding to non-heated OT in all three PAGES (Fig. 2). The precipitate fraction gave no band in native PAGE (Fig. 2A). On SDS-PAGE under non reducing conditions (Fig. 2B), the precipitate fraction gave band of OT aggregates remaining at the top of stacking gel, except for a main band corresponding to monomeric OT. Upon treatment with 2-ME, most of the protein was dissociated in a monomeric state (Fig. 2C). The intensity of monomeric band of OT solubilized with SDS plus 2-ME was higher than that with SDS alone (Fig. 2B, C).

In order to clarify the binding forces involved in the OT coagulation, soluble protein was extracted with each of four solutions (A–D) from the precipitates and then applied to SDS-PAGE under reducing conditions (Fig. 3). The intensities of band of OT in the extracts with solutions C and D were higher for comparison to those with the other solutions. That is, the larger quantities of OT were solubilized with solutions containing SDS, and almost all coagula were apparently dissolved with solution D. These results showed that the cooperative actions of NaCl and DTT in the presence of SDS as a main effective component were important for the solubilization of OT coagula, and that the binding forces contributing to OT coagulation were attributed to the cooperative actions of non-covalent forces such as hydrophobic interactions mainly and disulfide bonds partly. It has been reported that OT has 30 half-cystine residues per molecule, and that all these residues are

included in the intramolecular disulfide linkages (Mann, Fish, Cox, & Tanford, 1970). However, our electrophoresis experiments with heated OT indicated that disulfide linkages might be involved partly in the intermolecular aggregation. Kaneko and Kitabatake (1999) recently demonstrated that thaumatin containing eight intramolecular disulfide bonds and no cysteine residue makes precipitate through SH-catalyzed disulfide interchange among thaumatin molecules during heating at 80 °C for 15 min and pH 7.0. In our experiment with OT, free SH residues may be also produced through β -elimination of cystine residues during heating and may be involved in the intermolecular aggregation through SH-catalyzed disulfide interchange, as shown in the case of thaumatin, however, the appearance of SH residue in OT molecules during heating is uncertain.

3.2. Effects of OA on OT coagulation

The effects of OA on the heat coagulation of OT were determined by measuring turbidities of the mixed solutions after heating. The OT solution (0.05%; in the heating buffer, pH 7.0) was heated at 65 °C for 10 min with various OA concentrations (0–0.2%). As shown in Fig. 4, addition of increasing amounts of OA to OT resulted in decreased turbidity. The turbidity development was almost completely suppressed at 0.2% OA concentration. Thus, the heat coagulation of OT was found to be inhibited by the addition of OA in a concentration-dependent manner. In other words, clear solution can be prepared in the coexistence of OA even when OT solution was heated at 65 °C.

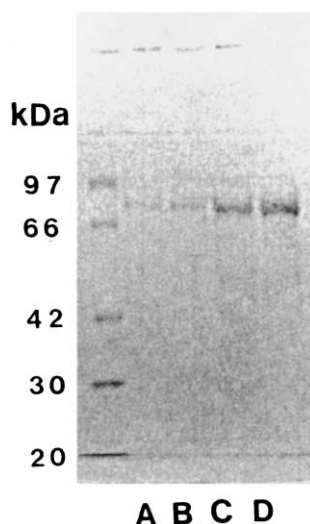


Fig. 3. SDS-PAGE analysis under reducing conditions of soluble fractions extracted from OT coagula by various solvents. The numbers in the figure indicate the molecular mass of marker proteins, as indicated in Fig. 2. A, 10 mM phosphate buffer (pH 7.0) with 0.6 M NaCl (solvent A); B, solvent A plus 1.5 M urea (solvent B); C, solvent A plus 0.5% SDS (solvent C); D, solvent A plus 0.5% SDS and 10 mM DTT.

In order to elucidate the possible mechanism for the inhibition by OA against OT coagulation, the mixed solution of OT (0.05%) with OA (0.2%) were heated at 65 °C for 10 min, and the resulting clear protein solution was analyzed with all three electrophoresis. In the native PAGE (Fig. 5A), some bands were observed at the top of separating gel, with the disappearance of OT and a slight decrease in intensities of OA bands. On SDS-PAGE under non-reducing conditions (Fig. 5B), the OA used in the experiment was found to already contain the dimer with the monomer of OA, as observed from the electrophoretic pattern of non-heated mixture of OT and OA. When the protein mixture was heated, soluble aggregates having higher molecular weights were observed on the each top of stacking gel and separating gel, with the disappearance

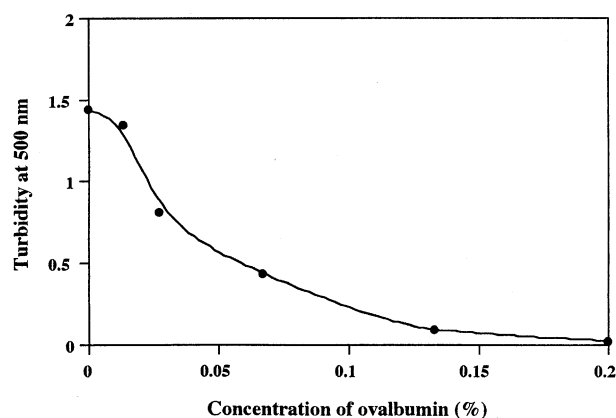


Fig. 4. Effect of ovalbumin (OA) on the turbidity development of OT solution (0.05%, pH 7.0) after heating at 65 °C for 10 min.

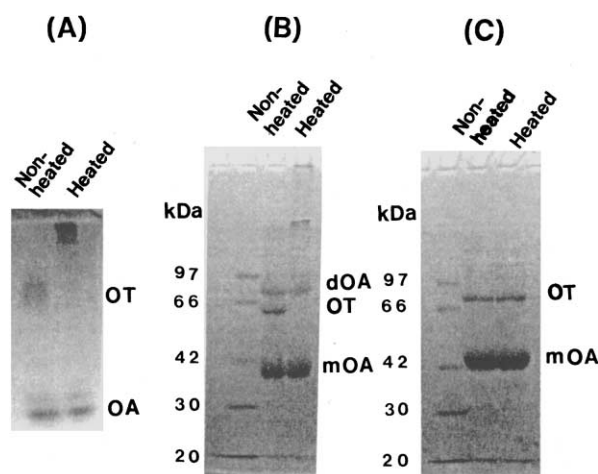


Fig. 5. Native PAGE and SDS-PAGE analysis of the mixed solution of OT (0.05%) and OA (0.2%) after heating at 65 °C for 10 min. dOA and mOA are the dimer and monomer of ovalbumin, respectively. The numbers in the figure indicate the molecular mass of marker proteins, as indicated in Fig. 2. (A), native PAGE; (B), SDS-PAGE under non-reducing conditions; (C), SDS-PAGE under reducing conditions.

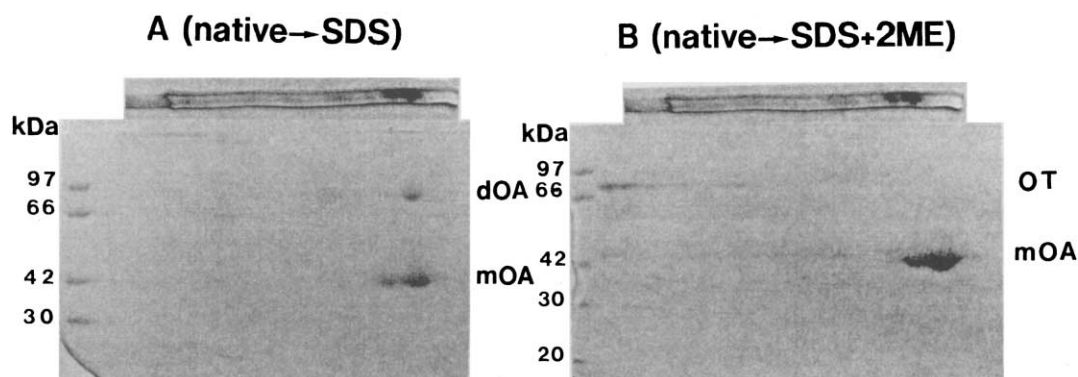


Fig. 6. Bi-dimensional electrophoretic analysis of the mixed solution of OT (0.05%) and OA (0.2%) after heating at 65 °C for 10 min. The first-dimension gel was native PAGE without SDS and reducing agent. A and B of the second-dimension gels were SDS-PAGEs under non-reducing conditions and reducing conditions, respectively. dOA and mOA are the dimer and monomer of ovalbumin, respectively. The numbers in figure indicate the molecular mass of marker proteins, as indicated in Fig. 2.

of OT, and the intensity of each band corresponding to dimer and monomer of OA also decreased slightly. In the presence of SDS and 2-ME, most soluble aggregates were dissociated into the monomeric OT and OA (Fig. 5C). These results indicated that disulfide bonding was contributed to the formation of soluble aggregates that were produced concomitantly with the disappearance of OT upon heating. To elucidate the behavior of OT heated in the coexistence of OA, the heated protein mixture was analyzed by bi-dimensional electrophoresis. Fig. 6 shows the first- and second-dimension patterns. In the first dimension gel portion (native PAGE) treated with the treatment buffer without reducing agent, the bands on the top of the separating gel did not migrate in the second dimension of 10% SDS-polyacrylamide gel, while the dimer and monomer of OA were observed in the second dimension gel in which each band of OA corresponded to the heterogeneity due to the extent of phosphorylation (Fig. 6A). When the first dimension gel was treated with the treatment buffer containing 2-ME, the bands on the top of the separating gel did migrate in the second dimension gel and dissociated into OT and OA (Fig. 6B). The result suggests that OT and OA interacted mutually during heating, and then produced the soluble aggregates that were composed of OT and OA through disulfide linkage. The formation of such aggregates might inhibit the OT-OT interaction that led to the coagulation upon heating.

3.3. Effect of NaCl on the inhibiting capacity of OA

The effect of added NaCl on the inhibiting capacity of OA against heat coagulation of OT was determined from the change in turbidity. The mixed solutions of OT (0.05%) and OA (0.1%) in the heating buffer (pH 7.0) containing various NaCl concentrations were heated at 65 °C for 10 min, the result of the turbidity development

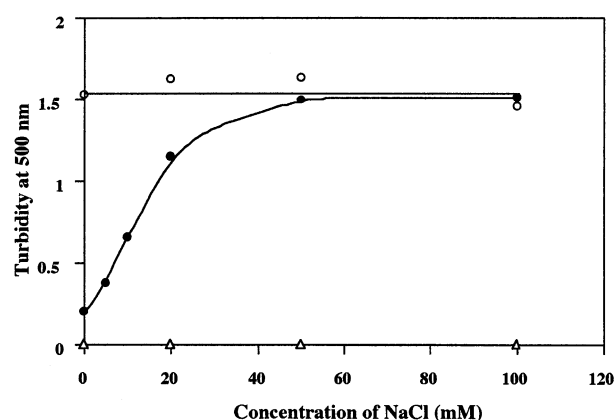


Fig. 7. Effect of NaCl on the turbidity development of the mixed solution of OT (0.05%) and OA (0.1%). Protein solutions (in 10 mM phosphate buffer, pH 7.0) were heated in the presence of various NaCl concentrations at 65 °C for 10 min, and the turbidity was measured. ○, OT alone; △, OA alone; ●, mixture of OT and OA.

being shown in Fig. 7. The turbidity of OT alone was independent on the NaCl concentration, maintaining the values of about 1.5. On the other hand, when 0.1% OA was heated separately, no precipitation was observed in any concentrations of NaCl used in this experiment. In the mixed solution of OT and OA, the turbidity gradually increased with an increase of added NaCl concentration, the development of turbidity recovering almost completely with 50 mM NaCl. The inhibition capacity of OA against OT coagulation was found to be weakened by the addition of NaCl. Because the major non-covalent force that is affected in the range below 0.2 M ionic strength is the electrostatic interaction (Eagland, 1975), the result suggested that the mode of interaction between OA and OT was electrostatic, that is, the electrostatic attraction between OA and OT would also contribute to the inhibition by OA against OT coagulation.

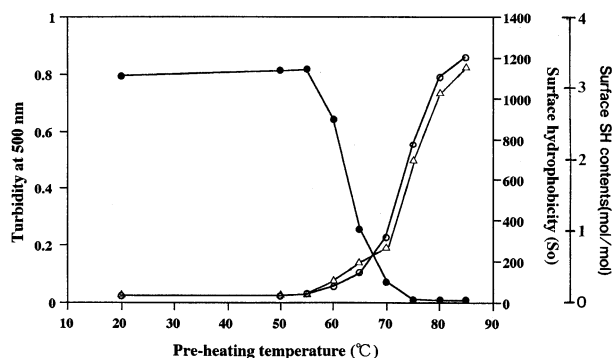


Fig. 8. Effect of the preheated OA on the turbidity development of OT solution, and the changes in surface hydrophobicity and surface SH residues of the preheated OA. The OA solution (0.2%, in the heating buffer, pH 7.0) was pre-heated for 10 min at various temperatures. The turbidity development of the mixture of OT (0.05%) and the preheated OA (0.02%) was measured after reheating for 10 min at 65°. ●, turbidity; ○, hydrophobicity; △, surface SH residue.

3.4. Effect of heat-denaturation of OA on the inhibiting capacity

OA might be denatured during heat treatment at 65 °C for 10 min and at pH 7.0. The partially unfolded conformation may be attributed to the inhibiting capacity of OA against heat coagulation of OT. Therefore, the characterization of OA is important for elucidating the inhibiting effect. The changes of OA solution (0.2%, in the heating buffer, pH 7.0) that occurred in the heat treatment at 65 °C for 10 min were examined. No differences in the CD spectra were observed between non-heated OA and heated OA, indicating no changes in the gross secondary structure (data not shown). On the other hand, the change in relative surface hydrophobicity was observed when the protein was heated at 65 °C. The S_0 values for non-heated OA and heated OA were obtained approximately as 30 and 150, respectively. In addition, the surface SH residues of OA apparently increased to 0.5 mole per mole of protein after heating at 65 °C. The SH residues in OA exist in the interior of the protein molecules and are exposed with heat denaturation (Matsudomi et al., 1986). These results indicated that some structural changes with the exposure of SH residues and hydrophobic residues in OA molecules occurred even after heating at 65 °C. Such structural changes on surface of OA molecules may be responsible for inhibition against heat coagulation of OT. In order to clarify the structural elements of OA contributing to the inhibition, the effects of heat denaturation of OA on the inhibiting capacity were investigated. The OA solution (0.2%, in the heating buffer, pH 7.0) was preheated at various temperatures for 10 min, and the inhibiting capacity of the preheated OA (0.02%) was examined by reheating at 65 °C for 10 min in the presence of OT (0.05%, in the heating buffer,

pH 7.0). Fig. 8 shows the correlation between the effect of preheated OA on the turbidity development of OT solution and S_0 values together with surface SH residues of the preheated OA. The turbidity development of OT in coexistence of the preheated OA after the reheating was reduced rapidly with the rise of preheating temperature from 60 to 75 °C for OA, and above 75 °C it reached a constant value of almost 0, that is, the mixture became transparent. Interestingly, such inhibiting capacities of preheated OA apparently showed a good correlation to the increments of S_0 values and surface SH residues in the protein.

4. Conclusion

In this study, the heat-induced interactions between OA and OT were investigated mainly from the viewpoint of the inhibition of OA against coagulation of OT. It was presumed that the inhibiting capacity of OA would be induced from some structural changes of OA molecules that occurred during heating. The preheating OA above 70 °C enhanced greatly the inhibiting capacity of OA against the heat-induced coagulation of OT. A possible mechanism by which OA inhibits the OT coagulation was proposed as follows: OA and OT interacted mutually by an electrostatic attraction at a low ionic strength and at pH 6.5. During heating, both heat-denatured proteins formed the soluble aggregates through hydrophobic interaction and disulfide linkages, and the formation of soluble aggregates that were composed of OA and OT suppressed the OT-OT interaction that led to coagulation. OA denatures and then forms soluble OA polymer upon heating, accompanying the increments of S_0 and surface SH groups as shown in this study. The extent of polymerization of OA might also affect the heat coagulation of OT. Additional study is in progress to elucidate the detailed mechanism of the inhibition by OA against OT coagulation.

References

- Careche, M., Alvarez, C., & Tejada, M. (1995). Suwari and kamaboko sardine gels: effect of heat treatment on solubility of networks. *Journal of Agriculture and Food Chemistry*, 43, 1002–1010.
- Cunningham, F. E., & Lineweaver, H. (1967). Inactivation of lysozyme by native ovalbumin. *Poultry Science*, 46, 1471–1477.
- Davis, B. J. (1964). Disc electrophoresis. 2. Method and application to human serum proteins. *Annals of New York Academy of Science*, 121, 240–244.
- Eagland, D. (1975). In: Duckworth, R. *Water relations of foods* (pp. 73–80). Academic Press, New York.
- Ellman, G. T. (1959). Tissue sulphhydryl groups. *Archives of Biochemistry and Biophysics*, 82, 70–75.
- Kaneko, R., & Kitabatake, N. (1999). Heat-induced formation of intermolecular disulfide linkages between thaumatin molecules that do not contain cysteine residues. *Journal of Agricultural and Food Chemistry*, 47, 4950–4955.

- Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta*, 624, 13–20.
- Kauzmann, W. (1959). Some factors in the interpretation of protein denaturation. In: *Advances in protein chemistry* (vol. 14, pp. 1–64). Academic Press, New York.
- Kekwiche, R. A., & Cannan, R. K. (1936). The hydrogen ion dissociation curve of the crystalline albumin of the hen's egg. *Biochemical Journal*, 30, 227–232.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, 227, 680–685.
- Mann, K. G., Fish, W. W., Cox, A. C., & Tanford, C. (1970). Single-chain nature of human serum transferrin. *Biochemistry*, 9, 1348–1354.
- Matsuda, T., Watanabe, K., & Sato, Y. (1981). Heat-induced aggregation of egg white proteins as studied by vertical flat-sheet polyacrylamide gel electrophoresis. *Journal of Food Science*, 46, 1829–1834.
- Matsuda, T., Watanabe, K., & Sato, Y. (1982). Interaction between ovomucoid and lysozyme. *Journal of Food Science*, 47, 637–641.
- Matsudomi, N., Takasaki, M., & Kobayashi, K. (1991). Heat-induced aggregation of lysozyme with ovotransferrin. *Agricultural and Biological Chemistry*, 55, 1651–1653.
- Matsudomi, N., Yamamura, Y., & Kobayashi, K. (1986). Heat-induced aggregation between ovalbumin and lysozyme. *Agricultural and Biological Chemistry*, 50, 1389–1395.
- Nakamura, R., & Matsuda, T. (1983). A new protein band appearing in the electrophoretic pattern of egg white heated at below 60 °C. *Journal of Food Science*, 48, 87–91.
- Wall, J. S., & Huebner, F. R. (1981). Adhesion and cohesion. In J. P. Cherry (Ed.), *Protein functionality in foods (ACS Symposium Series 147, chapter 6)* (pp. 111–130). Washington, DC: American Chemical Society.
- Yamashita, H., Ishibashi, J., Hong, Y.-H., & Hirose, M. (1998). Involvement of ovotransferrin in the thermally induced gelation of egg white at around 65 °C. *Bioscience, Biotechnology, and Biochemistry*, 62, 593–595.