JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Ability of αs-Casein to Suppress the Heat Aggregation of Ovotransferrin

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The effects of αs-casein on heat aggregation of ovotransferrin (OT) were studied by heating at 80 °C for 20 min in 10 mM phosphate buffer, pH 7.0. The heat interactions between αs-casein and OT were followed by turbidity development and polyacrylamide gel electrophoresis. We found that αs-casein can effectively suppress the heat-induced aggregation of heat-labile OT. The suppressive ability of αs-casein was reduced by the presence of NaCl on heating. Dephosphorylated αs-casein had less ability to suppress the aggregation of OT than native αs-casein. Our results indicate that αs-casein interacts with the heat-denatured OT through its exposed hydrophobic surface and phosphoserine residue. Such interactions seem to be important in helping to suppress the aggregation of heated OT. The suppressive effects of αs-casein on heat aggregation of OT would be partially ascribed to the formation of transparent gel from egg white by the addition of αs-casein.

KEYWORDS: αs-Casein; ovotransferrin; heat aggregation; dephosphorylation; ionic interaction; amphiphilic property

INTRODUCTION

Heat-induced gelation of egg white (EW) is one of its important functional properties with respect to EW usage in food systems (*1*). The gel properties of EW are sensitively affected by various factors, including pH, ionic strength, and salts (*2*−*5*). Most commercially available EW gives turbid products on heating. Thus, the nature of EW may not always be suitable for food materials. Hence, we have been studying the preparation of transparent gel from EW (*6*, *7*) and have recently reported that a transparent and firm gel can be prepared from EW by adding αs-casein on heating for gelling, indicating that EW proteins and αs-casein interact each other during gelling (*8*). Then, to clarify the formation of a transparent gel from EW by the addition of αs-casein, we have investigated the effects of αs-casein on the heat-induced aggregation of EW proteins.

Many investigators have extensively studied the heat-induced aggregation properties of EW proteins (*9*−*13*). Ovotransferrin (OT) is the most heat-labile protein in EW proteins and heating of EW even at a lower temperature near 60 °C caused the aggregation of OT (*14*−*16*). Such a high heat sensitivity of OT may be attributable to the formation of milky white gel from EW. We have found that αs-casein can suppress aggregation of dilute OT solution heated at 80 °C for 20 min at pH 7. In this study, we examine the factors contributing to the ability of αs-casein to suppress heat aggregation of OT, and then discuss a possible mechanism by which αs-casein suppresses the OT aggregation on the molecular basis.

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MATERIALS AND METHODS

Materials. Ovotransferrin (from chicken egg white that was substantially iron-free; OT), αs-casein (min 85%, from bovine milk), and β-casein (min 90%, from bovine milk) were purchased from Sigma Chemical Co. (St. Louis, MO). These protein samples were used without further purification. Acid phosphatase (from wheat germ) and trypsin treated with *N*-tosyl-L-phenylalanyl chloromethyl ketone (from bovine pancreas; TPCK-trypsin) was obtained from Sigma Chemical Co. The other chemicals were of reagent grade.

Preparation of the Phosphopeptide from β-Casein. Preparation of the peptide 1−25 from bovine β-casein was carried out by the procedure of Manson and Annan (*17*) with some modifications, that is, a solution of β-casein in water (1 g of protein in 50 mL) was digested at 20 °C and pH 8 for 4 h with TPCK-trypsin, using an enzyme−substrate ratio of 1:1000 (w/w), and then the peptide fraction was purified finally by Bio-Gel P-10 column chromatography, according to the method described by Otani et al. (*18*).

Heat Treatment and Measurement of Turbidity. Protein solutions (2 mL) in 10 mM sodium phosphate buffer, pH 7.0 (heating buffer) were heated at 80 °C for 20 min in a sealed glass vial (Pyrex culture tubes, Corning 9826). After the vials containing protein sample were heated, the absorbance of the sample was measured at 500 nm using a Hitachi spectrophotometer (model U-2000, Tokyo) and was used as the value for the turbidity (an indication of protein aggregation). The effect of αs-casein on heat aggregation of OT (0.5 mg/mL) was determined by comparison with heat aggregation in a single

10.1021/jf030802o CCC: $27.50
Published on Web 07/01/2004

system of OT or αs-casein. The effect of NaCl on the ability of αs-casein to suppress OT aggregation was determined by measuring the turbidity after the heat treatment in the heating buffer containing various concentrations of NaCl (0∼100 mM). Each data value of protein aggregation as measured by the turbidity at 500 nm in a 1-cm cuvette represents means of triplicate determinations.

Gel Electrophoresis. Native polyacrylamide gel electrophoresis (native PAGE) without sodium dodecyl sulfate (SDS) and reducing agent was carried out in 7.5% polyacrylamide gel sheet, as described by Davis (*19*). Bi-dimensional gel electrophoresis (native → SDS + 2-mercaptoethanol (2-ME)) was performed as follows: the first dimension was carried out in native PAGE as described above. After the run, the first-dimension gel portion was treated with 10 vol of the treatment buffer (62.5 mM Tris−HCl, pH 6.8, 1% SDS, 20% sucrose) 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 15% SDS-polyacrylamide gel according to the method of Laemmli (*20*). The protein bands were visualized by Coomassie blue staining. Molecular weight protein standards (phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; aldorase, 42 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; lysozyme, 14 kDa; Daiichi Pure Chemicals, Tokyo) were applied on SDS gel for comparison.

Dephosphorylation of αs-Casein. Dephosphorylation of αs-casein was carried out by means of acid phosphatase in 0.1 M acetate buffer (pH 5.5) containing 0.2% sodium azide, according to the method of Otani et al. (*18*) with some modifications. A 3-mg sample of the phosphatase was added to 100 mL of the 0.15% αs-casein solution dissolved in the buffer. The mixture was incubated in a water bath at 37 °C for various periods of time. The same protein sample was treated without the phosphatase under the same conditions for comparison. The reaction mixture was dialyzed against distilled water to remove the phosphate produced, and lyophilized. On the other hand, alkaline treatment of αs-casein solution (0.1%) was carried out in 0.3 M NaOH at 35 °C for 24 h. After the reaction, a sample was neutralized and lyophilized after dialysis against distilled water.

Determination of Phosphorus Content. The total phosphorus content of dephosphorylated αs-casein was determined by the method of Meum and Smith (*21*) after wet-ashing in sulfuric acid.

RESULTS AND DISCUSSION

Effects of αs-Casein on Heat Aggregation of OT. We have already reported that OT solution get turbid because of the formation of insoluble aggregates over the pH range 6.5∼8.5 upon heating at 65 °C, and the maximum turbidity was observed at about pH 7 (*13*, *16*). The OT solution (0.5 mg/mL; in the heating buffer, pH 7.0) was heated at 80 °C for 20 min with various concentrations of αs-casein (0−1 mg/mL). **Figure 1** shows the changes in the apparent absorbance at 500 nm of the solution of OT heated in the presence of αs-casein. In the absence of αs-casein, OT at the temperature of 80 °C undergoes denaturation followed by aggregation. On the other hand, when αs-casein solution was heated separately, the solution was still transparent at any concentrations of the protein used in this experiment (data not shown). In the presence of αs-casein, however, aggregation of heated OT was suppressed. Approximately 50% suppression was found at a 1:<0.2 (w/w) ratio of OT/αs-casein. Complete suppression of aggregation occurred at a 1:0.6 (w/w) ratio of OT/αs-casein, corresponding to a mole ratio of about 1:2. Thus, it seems likely that αs-casein can

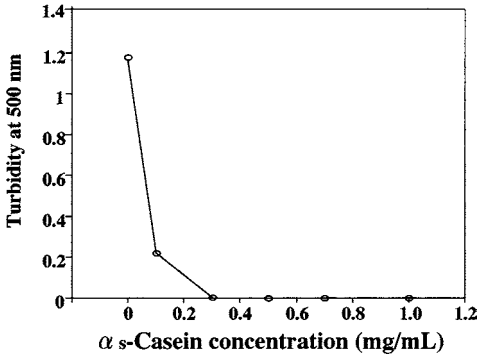

Figure 1. Turbidity of the solutions of OT (0.5 mg/mL) with various concentrations of αs-casein following heating at 80 °C for 20 min and pH 7.

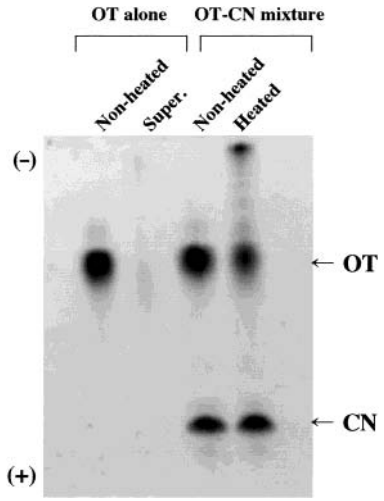

Figure 2. Native PAGE analysis of the solutions of OT (0.5 mg/mL) with and without αs-casein (0.3 mg/mL) following heating at 80 °C for 20 min and pH 7. OT and CN in the figure show ovotransferrin and αs-casein, respectively.

suppress the heat aggregation of OT in a concentration dependent manner. The result suggests that αs-casein and OT interact to suppress protein aggregation, and that the suppression of αs-casein against OT aggregation may contribute to the formation of clear gel from EW in the coexistence of αs-casein. Of interest in this result is the possible interaction between OT and αs-casein. It is conceivable that αs-casein enhances the solubility of denatured OT and stability of OT during heating.

We have revealed that heat-induced aggregation of OT was caused by a combination of hydrophobic interaction as well as thiol−disulfide interchange reaction (*16*). To clarify a possible mechanism for the suppression of αs-casein against OT aggregation, the dilute mixture of OT (0.5 mg/mL) and αs-casein (0.3 mg/mL) were heated at 80 °C for 20 min, and the resulting clear protein solution was analyzed by a native PAGE. In addition, OT solution (0.5 mg/mL) was heated at the same conditions, and then the obtained supernatant fraction after removal of insoluble OT aggregates by centrifugation was also applied to the native PAGE. The results are shown in **Figure 2**. When OT solution was heated, most of OT produced insoluble aggregates, and the supernatant fraction gave a faint band at the position corresponding to nonheated OT. On the mixed solution, some bands of soluble aggregates were observed at the top of separating gel, with a decrease in the intensity of OT monomer band, while αs-casein was dissociated almost completely, and it did not show apparent difference in the intensity

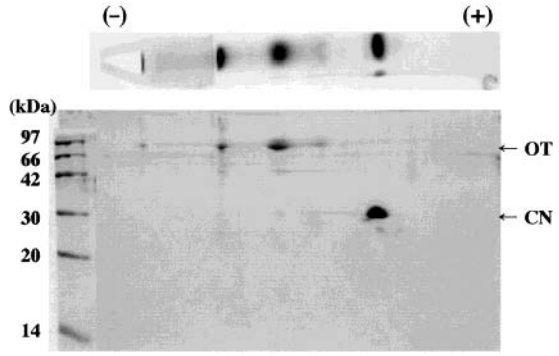

Figure 3. Bi-dimensional electrophoretic analysis of the solution of OT (0.5 mg/mL) with αs-casein (0.3 mg/mL) following heating at 80 °C for 20 min and pH 7. The first-dimension gel was native PAGE without SDS and reducing agent. The second-dimension gel was SDS–PAGE under reducing conditions. OT and CN in the figure show ovotransferrin and αs-casein, respectively, and the numbers indicate the molecular mass of marker proteins.

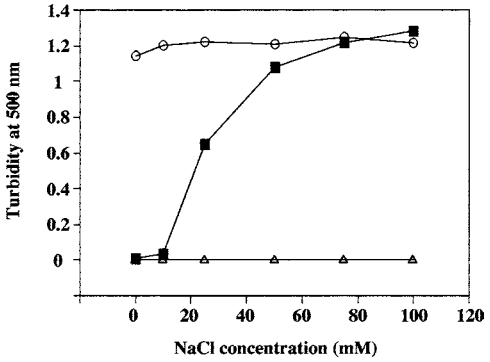

Figure 4. Turbidity of the solutions of OT (0.5 mg/mL) with and without αs-casein (0.3 mg/mL) in various concentrations of NaCl following heating at 80 °C for 20 min and pH 7. ○, OT alone; △, αs-casein alone; ■, mixture of OT and αs-casein.

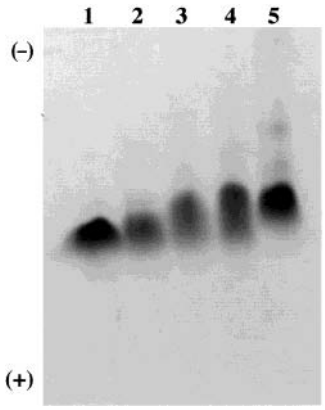

Figure 5. Native PAGE analysis of αs-caseins treated with phosphatase for various periods of time (h) and with alkali. Lane 1, control (treated for 9 h without phosphatase); lane 2, 0.5 h; lane 3, 3 h; lane 4, 9 h; lane 5, alkali-treated. The degree of dephosphorylation by phosphatase treatment for 0.5, 3, and 9 h was 50, 58, and 70%, respectively, and that of the alkaline hydrolysate was 90%.

of the protein band before and after heating. The result suggests that αs-casein might interact with OT on the base of ionic interaction during heating. To further elucidate the behavior of OT heated with αs-casein, the bi-dimensional electrophoresis of the heated protein mixture was carried out. As shown in **Figure 3**, in the first dimension gel portion (native PAGE) treated with the treatment buffer containing 2-ME, some bands on the top of the separating gel did migrate in the second dimension gel and were dissociated into OT only. The result indicates that OT heated with αs-casein produced soluble OT aggregates in part, in addition to OT monomer. From the result of the experiment, it was considered that αs-casein did not bond covalently to OT but prevented OT from aggregating. Alternatively, as αs-casein is a highly hydrophobic and protein with more stretched structure, it may have a tendency to interact instantly with the exposed hydrophobic groups of denatured OT. Thus, it was suggested that αs-casein may create a nonreactive barrier by placing itself between the heat-denatured OT molecules and may suppress OT−OT interaction that led to insoluble aggregates.

Effect of NaCl on the Suppressive Ability of αs-Casein. The effect of NaCl on the ability of αs-casein to suppress heat aggregation of OT was determined by following the change in turbidity. The mixed solutions of OT (0.5 mg/mL) and αs1-casein (0.3 mg/mL) in the heating buffer (pH 7.0) containing various concentrations of NaCl (0−100 mM) were heated at 80 °C for 20 min, with the result of the turbidity development being shown in **Figure 4**. The turbidity development of OT solution (0.5 mg/mL) alone was independent of the NaCl concentration, maintaining the values of about 1.2 on the absorbance at 500 nm at all NaCl concentrations used in this experiment. On the other hand, when αs-casein solution (0.3 mg/mL) was heated separately, the solution was still transparent at all NaCl concentrations tested. In the mixture of OT and αs-casein, the turbidity gradually increased with an increase of NaCl concentration, the development of turbidity recovering almost completely with 75 mM NaCl. Thus, the ability of αs-casein to suppress OT aggregation was weakened by the presence of NaCl. Because the major noncovalent force that is affected in the range below 0.2 M ionic strength is an electrostatic interaction (*22*), our result suggested that the suppression of OT aggregation by αs-casein takes place through ionic interactions. Such interactions might be due to the action of phosphoserine residues in αs-casein as an anion.

Effect of Dephosphorylation of αs-Casein on the Suppressive Ability. Dephosphorylated αs-casein was prepared by the treatment with phosphatase and with alkali. **Figure 5** shows a native PAGE pattern of the hydrolysates treated with the phosphatase for an indicated time and alkaline-treated one. On the electrophoretic analysis, a broad band having lower mobilities was observed in their hydrolysates. The low electrophoretic mobility of the band reflects the degree in negative charge resulting from dephosphorylation of the protein. It was estimated by the method of Meun and Smith (*21*) that the degree of dephosphorylation by phosphatase was about 50% for 0.5 h-hydrolysate, 58% for 3 h-one, and 70% for 9 h-one. Thus, because phosphate residues in αs-casein were not completely removed by the phosphatase treatment, dephosphorylation with alkali was carried out. In the case of alkaline treatment, the release of phosphate residues was found to be about 90%. **Figure 6** shows the suppressive abilities of the enzymatic and alkaline hydrolysates in comparison with that of native αs-casein against heat aggregation of OT. The suppressive ability of αs-casein was reduced with the degree of removal of negatively charged phosphate residues, indicating that phosphoserine residues contribute to the suppression of OT aggregation. When the degree of dephosphorylation of αs-casein exceeded 70%, turbidity of the solution of OT (0.5 mg/mL) heated with these caseins exhibited a minimum value at casein concentration of 0.3 mg/mL and then gradually increased at high casein to OT

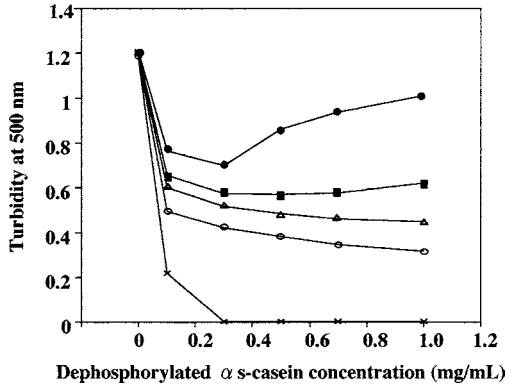

Figure 6. Turbidity of the solutions of OT (0.5 mg/mL) with various concentrations of dephosphorylated αs-casein following heating at 80 °C for 20 min and pH 7. The dephosphorylation of αs-casein was done by the treatments with phosphatase for various periods of time (h) and with alkali, as shown in **Figure 5**. ×, control; ○, 0.5 h; △, 3 h; ■, 9 h; ●, alkali-treated.

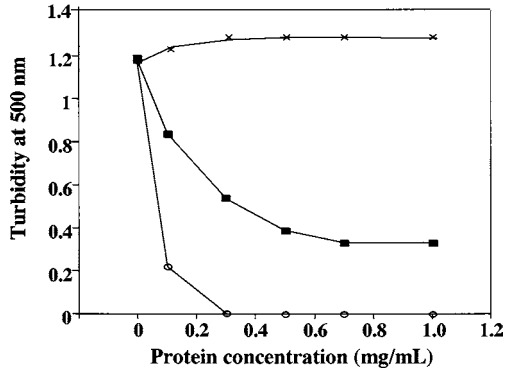

Figure 7. Comparative abilities of αs-casein, β-casein, and the phosphopeptide (peptide 1–25 from β-casein) to suppress heat aggregation of OT solution (0.5 mg/mL). ○, αs1-casein; ■, β-casein; ×, phosphopeptide.

ratio. At high casein to OT ratio, such an increment of turbidity may be due to the self-association of a highly dephosphorylated αs-casein as a result of loss of amphiphilic property. On the other hand, 90% dephosphorylated αs-casein still exhibited very little the suppressive ability. Therefore, we assumed that other factors besides phosphoserine residue seem to contribute to the suppression of OT aggregation. One possible explanation may be that the combined action of phosphoserine residue and hydrophobic region in αs-casein may be important for suppressing heat aggregation of OT.

Comparative Abilities of αs-, β-Caseins, and the Phosphopeptide (Peptide 1−25 from β-Casein) to Suppress the Heat Aggregation of OT. αs- and β-Caseins are a family of phosphoproteins having distinct hydrophilic and hydrophobic regions and random coil structure. A common feature of these caseins is the propensity to form in nature a micellar structure due to the amphiphilic property. Such characteristics might have a bearing on their ability to interact with denatured OT. The abilities of β-casein and the phosphopeptide (peptide 1−25 from β-casein) to suppress the heat aggregation of OT were compared with those of αs-casein, as shown in **Figure 7**. β-Casein suppressed moderately in comparison to αs-casein, although the phosphopeptide derived from β-casein showed no measurable suppression. The phosphopeptide is a highly hydrophilic because of the existence of a phosphoserine-rich sequence (residues 15−19) in β-casein (*23*). Thus, such a complete loss of suppressive ability of the phosphopeptide may be due to a result of the deficiency of amphiphilic property. To exhibit the suppressive ability, αs-casein would appear to require an amphiphilic structure having hydrophobic region besides highly phosphorylated core region that favors the suppression of OT aggregation. It is not immediately clear how the addition of αs-casein to OT solution suppressed the heat aggregation of OT. One possibility is that αs-casein interacts with the exposed hydrophobic surface of heat-denatured OT, and then the polyanion on the surface of the hydrophobically bonded αs-casein−OT complex prevents the coalescence of the complex by their repulsive electrostatic forces, thus preventing OT−OT interaction that led to insoluble aggregates. The structure−function relationship of αs-casein on OT aggregation is being investigated in detail using some protease-treated αs-casein.

Conclusion. αs-Casein had the high ability to suppress heat aggregation of OT. Hydrophobic region and phosphoserine residues in αs-casein may be essential for the suppression of OT aggregation. The formation of transparent gel from egg white by the addition of αs-casein seems to be caused partly by the suppression against heat aggregation of OT by αs1-casein.

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Received for review December 8, 2003. Revised manuscript received April 27, 2004. Accepted May 13, 2004.

JF030802O