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Phosphorylation of Ovalbumin by Dry-Heating in the Presence of Pyrophosphate: Effect on Protein Structure and Some Properties

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Ovalbumin (OVA) was phosphorylated by dry-heating in the presence of pyrophosphate at pH 4.0 and 85 °C for 1 and 5 days, and the physicochemical and structural properties of phosphorylated OVA were investigated. The phosphorus content of OVA increased to 1.01% by phosphorylation, and the electrophoretic mobility of PP-OVA also increased. Although the solubility of dry-heated OVA decreased, the decrease was slightly depressed by phosphorylation. The circular dichroism spectra showed that the change of the secondary structure in the OVA molecule, as measured by α-helix content, was mild by phosphorylation. The exchange reaction between the sulfhydryl and disulfide groups was enhanced and the surface hydrophobicity of OVA increased by phosphorylation. The tryptophan fluorescence intensity of OVA decreased by phosphorylation, suggesting that the conformational change occurred in the OVA molecule by phosphorylation. Although the differential scanning calorimetry thermograms of OVA showed a lowering of the denaturation temperature from 78.3 to 70.1 °C by phosphorylation, the stability of OVA against heat-induced insolubility at pH 7.0 was improved. The results indicated molten (partially unfolded) conformations of OVA formed by dry-heating in the presence of pyrophosphate.

KEYWORDS: Ovalbumin; dry-heating; phosphorylation; structural properties; denaturation temperature; circular dichroism; differential scanning calorimetry

INTRODUCTION

Phosphorylation is a useful method for the improvement of functional properties of food proteins. Functional properties of some phosphorylated proteins have been studied and reviewed by Matheis and Whitaker (*1*). Woo and Richardson (*2*) reported that the emulsifying activity of phosphorylated β-lactoglobulin increased. Sitohy et al. (*3*) phosphorylated β-lactoglobulin with $POCl_3$ under mild conditions and succeeded in preparing proteins with few cross-linking molecules. Seguro and Motoki (*4*) phosphorylated soybean protein by an enzymatic method. Aoki et al. (*5*, *6*) and Kato et al. (*7*) reported that ovalbumin (OVA) and β-lactoglobulin were phosphorylated by conjugation of glucose-6-phosphate to protein through the Maillard reaction. However, there were some problems in these phosphorylation methods mentioned in previous papers (*8*, *9*). We have phosphorylated egg white protein (EWP) by dry-heating in the presence of phosphate (*8*, *9*), and the heat stability and emulsifying properties of EWP were improved. Furthermore, the calcium phosphate-solubilizing ability of EWP was enhanced by phosphorylation. Interestingly, a transparent and firmer heat-induced gel of phosphorylated EWP was obtained as reported in a previous paper (*8*).

Although the functional properties, especially the gelling properties, of EWP were improved by dry-heating in the presence of phosphate, the molecular basis of structural changes causing the improvement have not yet been unraveled sufficiently. OVA is the main constituent of EWP, and its behavior predominantly affects the functional properties of EWP. Therefore, monitoring the physicochemical and structural changes of phosphorylated OVA (PP-OVA) by dry-heating in the presence of pyrophosphate will be helpful for further understanding of the relationship between the structure and functional properties.

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In the present study, we phosphorylated OVA by dry-heating in the presence of pyrophosphate and described some physicochemical and structural properties of PP-OVA.

MATERIALS AND METHODS

Materials. Ovalbumin was purified from the fresh egg white protein in the following two steps: (1) precipitation with 50% saturated ammonium sulfate at pH 4.5 three times and (2) column chromatography with CM-cellulose (Whatman International Ltd., Maidstone, Kent, U.K.) equilibrated with 50 mM sodium acetate buffer, at pH 4.4, and the OVA fraction was eluted with a 50 mM sodium acetate buffer, at pH 4.9. 1-Anilino-8-naphthalenesulfonate (ANS) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). All other reagents were of analytical grade.

Preparation of PP-OVA and Dry-Heated OVA (DH-OVA). PP-OVA and DH-OVA were prepared according to the method given in a previous paper (*9*). OVA was dissolved at 2% in 0.1 M sodium pyrophosphate buffer at pH 4.0, adjusting the pH with 1 N HCl, and the solution was lyophilized. Lyophilized samples were incubated at 85 °C for 1 and 5 days. Dry-heated samples were dissolved and dialyzed to remove free pyrophosphate for 3 days against deionized water and then lyophilized.

In comparison with PP-OVA, DH-OVA was prepared as follows: OVA was dissolved at a concentration of 2% in deionized water and the pH of the solution adjusted to 4.0 with 1 N HCl; the mixture was then lyophilized and dry-heated under the same conditions as those of PP-OVA. Finally, dry-heated samples were dissolved and dialyzed for 3 days against deionized water and then lyophilized.

Determination of Phosphorus Content (P Content) of PP-OVA. Protein samples were digested in perchloric acid. Phosphorus in the digest was regarded as the total phosphorus of PP-OVA. For the determination of inorganic phosphorus (P_i), 5 mL of 10% trichloroacetic was added to the same volume of 1% PP-OVA solution, and the solution was centrifuged at 3000*g* for 20 min. The phosphorus in the supernatant was regarded as P_i. The P content was determined according to the method of Chen et al. (*10*). The amount of phosphorus bound to proteins was estimated by the difference between the total phosphorus and P_i content.

Electrophoresis. Native polyacrylamide gel electrophoresis (native-PAGE) was performed using 8% polyacrylamide gels in the absence of sodium dodecyl sulfate (SDS), and SDS-PAGE was performed using 10% polyacrylamide gels under both reducing and nonreducing conditions in the presence and absence of 2-mercaptethanol (2-ME) and SDS according to the method of Laemmli (*11*). The gels were stained in Coomassie Blue G-250 for 1 h.

Measurement of Sulfhydryl Thiol (SH) Groups. Free SH groups were determined using DTNB according to the procedure of Ellman (*12*). To 1 mL of 0.1% protein solution were added 1 mL of 0.1 M Tris−glycine buffer (pH 8.0) containing 0.01 M EDTA (for surface SH groups) and the same buffer containing 5% SDS (for total SH groups). 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. Absorbance was read at 412 nm. A molar extinction coefficient of 13600 M^{-1} cm^{-1} at 412 nm of 2-nitro-5-mercaptobenzoic acid was used for calculation of the modified sulfhydryl groups in the OVA molecule using DTNB. The SH content was calculated as moles of SH per mole of OVA.

Evaluation of Surface Hydrophobicity. The surface hydrophobicity of OVA was evaluated by measuring the fluorescence intensity (FI) and initial slope (S_0) of OVA in the presence of ANS according to the method of Hayakawa and Nakai (*13*). The OVA samples were dissolved in 20 mM phosphate buffer (pH 7.4), containing 0.1 mM EDTA to give 1% of OVA concentration, and then diluted with the same buffer for a series of three concentrations between 0.1 and 0.5%. One hundred microliters of a 0.3 g/L ANS solution in the same phosphate buffer was added to 2 mL of OVA solution as the fluorescence probe and then incubated at 25 °C for 1 h. FI was measured with an FP-6600 fluorescence spectrophotometer (Jasco Co., Tokyo, Japan) at an excitation wavelength of 370 nm and an emission wavelength of 470 nm at 25 °C. Emission spectra were corrected for background fluorescence caused by ANS in reactions lacking protein. The S_0 was calculated from the FI versus protein concentration plot.

Table 1. Some Characteristics of N-, DH-, and PP-OVA

protein	P content[a] (%)	SH content[b] (mol/mol) surface	SH content[b] (mol/mol) total	S_0[b,c]	solubility[b] (%)
N-OVA	0.12	0.07a ± 0.02	3.98a ± 0.05	30a ± 4	100.0a ± 0.4
DH-OVA-1d	0.12	0.14b ± 0.03	3.82b ± 0.01	40b ± 3	97.8a ± 1.2
DH-OVA-5d	0.12	0.20c ± 0.01	3.79b ± 0.03	45b ± 2	95.4b ± 1.1
PP-OVA-1d	0.64	0.19c ± 0.04	3.67c ± 0.04	66c ± 7	99.0a ± 1.0
PP-OVA-5d	1.01	0.52d ± 0.05	3.42d ± 0.02	129d ± 13	97.8a ± 0.8

[a] Data shown are the mean value of the two determinations, with a deviation of <0.01. [b] Each value is the mean with its SD ($n = 4$); means in same column with different letters are significantly different ($p < 0.05$). [c] S_0 was expressed as an initial slope of FI versus protein concentration (in percent) plot.

Measurement of Solubility. Protein samples were dissolved at a concentration of 0.1% in 50 mM Tris-HCl buffer (pH 7.0) and then centrifuged at 3000*g* for 20 min. The concentration of protein in the supernatant was determined using the method of Lowry et al. (*14*).

Circular Dichroism (CD) Spectra. CD spectra were measured at 190−260 nm with a Jasco J-720 spectropolarimeter (Jasco Co., Tokyo, Japan), and the digitized data were transferred to a microcomputer and processed. Samples were dissolved in 50 mM Tris-HCl buffer (pH 7.0) at a concentration of 0.1 g/L. CD spectra were represented in terms of mean residue ellipticity (degrees cm^2/dmol).

Tryptophan (Trp) Fluorescence Spectra. Trp fluorescence intensity of OVAs was scanned at emissions from 300 to 400 nm excited at a wavelength of 280 nm by an FP-6600 fluorescence spectrophotometer (Jasco Co.) at 25 °C. OVA sample was dissolved at a concentration of 0.06 g/L in a 50 mM phosphate buffer (pH 7.0).

Differential Scanning Calorimetry (DSC). DSC was performed in a VP-DSC Microcalorimeter (MicroCal, Northampton, MA). Prior to DSC experiments, samples were dialyzed against 20 mM phosphate buffer (pH 7.4). After being filtered through a 0.45 μm filter, samples and reference solutions were properly degassed and loaded into the calorimeter, and the experiments were carried out under an extra pressure of 1 atm to avoid degassing during heatings. The calorimetric data were analyzed using Origin software provided with the calorimeter. The protein concentration was 0.5 g/L and was heated in the calorimeter at a scan rate of 1 °C/min over the range 20−120 °C.

Measurement of the Stability of OVA against Heat-Induced Insolubility. Protein samples were dissolved at a concentration of 0.1% in 50 mM Tris-HCl buffer (pH 7.0). The sample solutions (2 mL) were placed in a small test tube with an aluminum foil stopper and were heated in a water bath at 60−95 °C for 10 min. Aggregates were precipitated by centrifugation at 3000*g* for 20 min. The soluble protein in the supernatant was measured according to the method of Lowry et al. (*14*) to estimate the protein concentration of the solution.

RESULTS AND DISCUSSION

Characteristics of PP-OVA. OVA was phosphorylated by dry-heating at pH 4.0 and 85 °C for 1 and 5 days. The P content of phosphorylated OVA is shown in **Table 1**. The P content of native OVA, which is naturally phosphorylated at serine-68 and/or -344, is 0.12% (*15*). However, this level of phosphorylation was considered to be too low to generate some of the functional properties of OVA. In the present study, we phosphorylated OVA by dry-heating at pH 4.0 and 85 °C for 1 and 5 days. The P content of OVA was 0.64% by dry-heating at pH 4.0 and 85 °C for 1 day and became 1.01% by dry-heating for 5 days, which was higher than that of bovine whole casein (*9*). **Figure 1A** shows the native-PAGE pattern of native (N-), DH-, and PP-OVA. Usually, N-OVA was dissociated into three bands (A_1, A_2, and A_3) depending on the number of phosphoserine residues (*16*). In the present study, only bands A_1 and A_2 could be clearly observed, and band A_3 was very weakly stained due to the

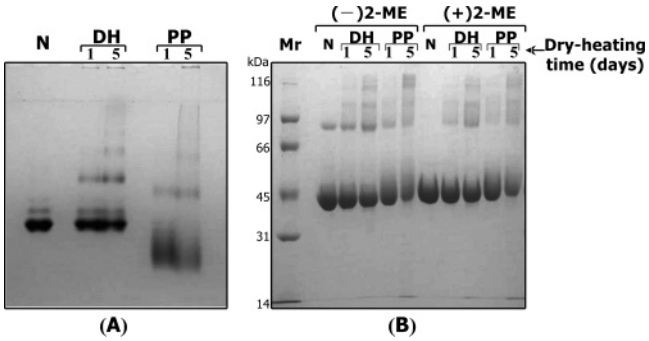

Figure 1. Electrophoretic patterns of N-, DH-, and PP-OVA: (**A**) native PAGE (8% polyacrylamide gel in the absence of SDS); (**B**) SDS-PAGE (10% polyacrylamide gel in the presence of 1.7% SDS) with (+) and without (–) 5% of 2-mercaptoethanol (2-ME). Mr, marker protein; N, native OVA; DH, OVA dry-heated in the absence of pyrophosphate; PP, OVA dry-heated in the presence of pyrophosphate.

smaller amount of this component (~3% of total OVA). In the absence of pyrophosphate, there were almost no changes in the mobility of OVA. However, the mobility of OVA increased by dry-heating in the presence of pyrophosphate, indicating that a higher level of negatively charged phosphate groups on OVA caused greater mobility. In the present paper, the phosphorylation level of PP-OVA is similar to that of PP-EWP reported in a previous paper (*8*) by dry-heating under the same conditions. From the native-PAGE pattern of OVA, the aggregation of OVA by dry-heating was observed, and the aggregation increased with the dry-heating time from 1 to 5 days (**Figure 1A**). To assess the binding type of aggregates, SDS-PAGE in the absence and presence of 2-ME, respectively, was performed. It can be clearly seen from **Figure 1B** that the dry-heating induced substantial aggregation in the protein, leading to the coexistence of different populations of monomeric and multimeric OVA. When OVA was dry-heated for 5 days in the absence and presence of pyrophosphate, the intensities of the bands of aggregates increased and the relative concentrations of OVA monomer decreased. In the presence of SDS and 2-ME, the dimer and oligomer of OVA were considerably reduced to monomers; however, most aggregates having higher molecular weights remained undissociated. The results indicated that not only disulfide bonds but also other types of bonds were formed by dry-heating. The covalent bonds other than disulfide bonds formed in proteins by dry-heating have been discussed by some researchers (*17*, *18*), but the structures of the covalent bonds have not yet been elucidated. It has been reported that cross-linking by amidation between carbonyl and ϵ-amino groups or by transamidation between such groups with the elimination of ammonia occurs on severe heat treatment in the protein molecule (*19*). Thus, such covalent bonds as mentioned above may be formed in OVA on dry-heating in the absence and presence of pyrophosphate. On the other hand, it was also observed that the mobility of PP-OVA was slightly slower than that of DH-OVA in the absence and presence of 2-ME, suggesting a slight increase of the molecular mass of OVA by phosphorylation.

The effect of the phosphorylation on the solubility of OVA was measured at pH 7.0. Although the solubility of OVA decreased slightly by dry-heating in the absence of pyrophosphate for 5 days, the insolubility of OVA was slightly depressed by phosphorylation (**Table 1**).

Effect of Phosphorylation on the Structure and the Stability against Heating of OVA. To determine the impact of the phosphorylation on the structural properties of the protein at a secondary folding level, CD spectroscopy was used. **Figure 2** shows the CD spectra of OVAs. N-OVA is known to have 31% α-helix, 27.5% β-sheet, 13.5% β-turn, and 28% random coil (*20*). α-Helix content can be estimated from the mean residue ellipticity at 222 nm (θ_{222}), using the equation

$$\alpha\mathrm{H} = (\theta_{222} - \theta_c)/(\theta_h - \theta_c)$$

where θ_h and θ_c are the mean residue ellipticity values for fully helical and random coil polypeptides, respectively, using the values of −36 000 and −260 in degrees cm^2/d mol, respectively (*21*). In the present paper, α-helix content for native OVA was 30.8% (**Table 2**), which was close to the result reported by Batra et al. (*20*). There was almost no change in the α-helix content of OVA by dry-heating for 1 day in the absence of pyrophosphate, but a slight decrease was observed by dry-heating for 5 days. A slight shift to long wavelength at the maximum value near 220 nm of CD spectra was observed by dry-heating in the absence and presence of pyrophosphate. This may be caused by aggregation of the OVA molecule at the α-helix region by dry-heating (*22*). In the presence of pyrophosphate, there was a slight decrease in the α-helix content of OVA by dry-heating for 1 day, which decreased further by dry-heating for 5 days, while maintaining its gross structure without major change. These results suggested that the secondary structure of OVA was not significantly affected by phosphorylation with pyrophosphate. Kato et al. (*10*) reported the secondary structure of OVA (CD spectra) was scarcely affected by dry-heating at 80 °C for 5 days. A similar result was also obtained by Matsudomi et al. (*23*). N-OVA contains four sulfhydryl (SH) groups and one disulfide (SS) group per molecule. The SH residues in OVA that exist in the interior of the protein molecules are exposed

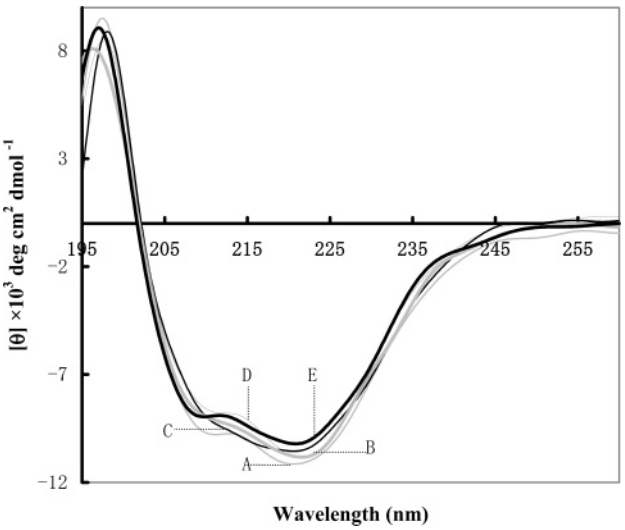

Figure 2. CD spectra of OVAs: (**A**) native OVA; (**B**, **C**) OVA dry-heated for 1 and 5 days in the absence of pyrophosphate; (**D**, **E**) OVA dry-heated for 1 and 5 days in the presence of pyrophosphate.

Table 2. α-Helix Content (αH) and Denaturation Temperature (T_d) of OVAs

protein	αH[a] (%)	T_d[b] (°C)
N-OVA	30.8	78.3
DH-OVA-1d	30.0	78.2
DH-OVA-5d	29.2	77.9
PP-OVA-1d	29.3	72.3
PP-OVA-5d	28.1	70.1

[a] Based on the CD spectra shown in **Figure 2**. [b] Based on the DSC profile shown in **Figure 5**.

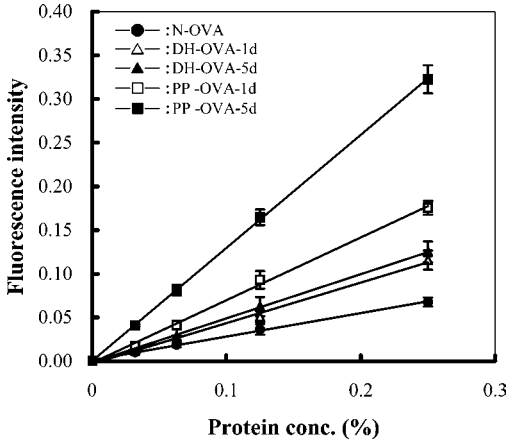

Figure 3. FI of N-, DH-, and PP-OVA measured by binding with ANS. N-OVA, native OVA; DH-OVA-1d and -5d, OVA prepared by dry-heating at pH 4.0 and 85 °C for 1 and 5 days in the absence of pyrophosphate; PP-OVA-1d and -5d, OVA prepared by dry-heating at pH 4.0 and 85 °C for 1 and 5 days in the presence of pyrophosphate. Each value is the mean with its SD ($n = 3$).

with heat denaturation. The changes in surface and total SH contents in OVA are shown in **Table 1**. The total SH content of N-OVA was 3.98 mol, which agrees well with the value (∼4 mol) of N-OVA reported by Nisbet et al. (*15*). The surface SH content of OVA increased by the dry-heating time from 1 to 5 days, and the phosphorylation by dry-heating in the presence of pyrophosphate enhanced the increase of surface SH of OVA. On the other hand, the total SH content of OVA decreased by dry-heating, and the phosphorylation enhanced the decrease of total SH content. These results suggested that exposure of SH groups to the surface on OVA molecules and then SH−SS exchange reactions and SH oxidation might proceed simultaneously by dry-heating. In addition, this was promoted further by dry-heating in the presence of pyrophosphate.

The effect of phosphorylation on the surface hydrophobicity of OVA was evaluated by measuring the FI of OVA using the ANS binding method. **Figure 3** shows FI of N-, DH-, and PP-OVA at protein concentrations from 0.1 to 0.5%. The FI of OVA increased by dry-heating in the absence of pyrophosphate and further increased greatly by phosphorylation. The S_0 of PP-EWP dry-heated at 85 °C and pH 4.0 for 5 days was 129, which was 4.2-fold over that of N-OVA (30) (**Table 1**). These results suggest that phosphorylation of OVA through dry-heating in the presence of pyrophosphate caused the more buried hydrophobic residues to become exposed rather than that of OVA dry-heated in the absence of pyrophosphate. In the present study, ANS was used as a probe for the measurement of surface hydrophobicity of EWP. According to Nakai and Li-Chan (*24*), ANS binds to aromatic amino acid side chains from aromatic amino acids such as Phe, Trp, and Tyr, whereas *cis*-parinaric acid (CPA) binds to aliphatic amino acid side chains from aliphatic amino acids such as Val, Leu, and Ile. Hydrophobicity measured with ANS shows a significant relationship with protein solubility, whereas hydrophobicity measured with CPA does not. In the present paper, although the remarkable increase in ANS surface hydrophobicity of PP-OVA was observed, most of the PP-OVA remained soluble at pH 7.0. This was considered to be due to the introduced negative charge of phosphate groups.

The Trp fluorescence spectra of OVA are recorded and shown in **Figure 4**. The Trp fluorescence of OVA was slightly decreased by dry-heating and further decreased by phosphorylation through dry-heating in the presence of pyrophosphate.

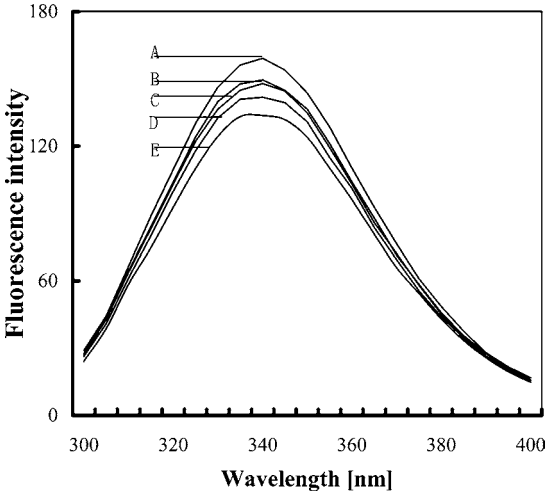

Figure 4. Trp fluorescence spectra of OVAs: (**A**) native OVA; (**B**, **C**) OVA dry-heated for 1 and 5 days in the absence of pyrophosphate; (**D**, **E**) OVA dry-heated for 1 and 5 days in the presence of pyrophosphate. The excitation wavelength was 280 nm, and the emission was scanned from 300 to 400 nm. Fluorescence spectra of samples were measured at 0.06 g/L in triplicate.

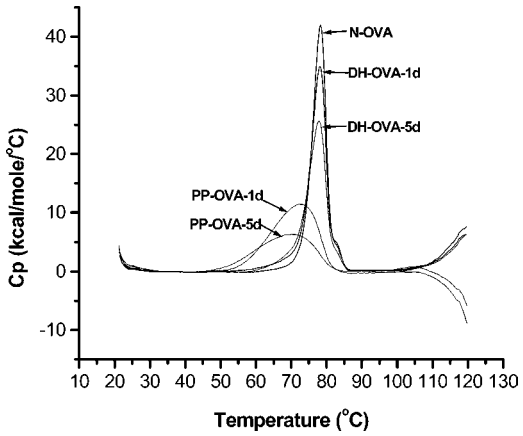

Figure 5. DSC profiles of N-, DH-, and PP-OVA. DSC scans were performed with protein solution of 0.5 g/L in 20 mM phosphate buffer at pH 7.4. The sample was heated in the calorimeter at a scan rate of 1 °C/min over the range 20–120 °C. N-, DH-, and PP-OVA: see **Figure 3**.

From these results, it was suggested that conformational changes induced more exposure of Trp residues to solvent by phosphorylation. The results from the SH−SS exchange reaction, surface hydrohobicity, and Trp fluorescence spectra also showed that the change in tertiary structure of OVA molecule was caused by phosphorylation.

To investigate the thermodynamic stability of PP-OVA, DCS experiments were carried out. From the thermograms shown in **Figure 5**, it can been seen that N-OVA has a denaturation temperature (T_d) of 78.3 °C with a symmetric band shape, and dry-heating for 1 and 5 days in the absence of pyrophosphate results in a slight lowering of the T_d (respectively, −0.4 and −0.9 °C) (**Table 2**). On the other hand, when OVA was phosphorylated by dry-heating for 1 and 5 days in the presence of pyrophosphate, there was a broadening of the peaks with a marked decrease in T_d values (respectively, −6.0 and −8.2 °C) (**Table 2**), revealing that the thermodynamic stability and denaturation temperature of OVA decreased by phosphorylation. This behavior of decrease of T_d was considered to be due to a relatively unfolded structure caused by electrostatic-repulsive force of phosphate groups in the OVA molecule. However,

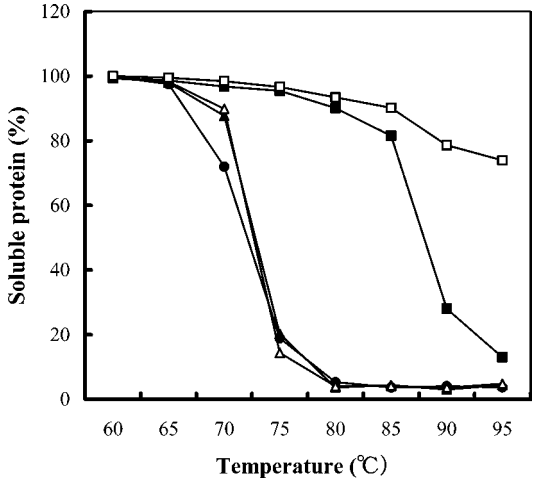

Figure 6. Stability against heat-induced insolubility of N-, DH-, and PP-OVA at various temperatures: (●) native OVA; (▲) DH-OVA-1d; (△) DH-OVA-5d; (■) PP-OVA-1d; (□) PP-OVA-5d. The protein sample was 1 g/L in 50 mM Tris-HCl buffer (pH 7.0) and heated at 60–95 °C for 10 min. Data shown are the mean value of the two determinations, with a deviation of <1%. N-, DH-, and PP-OVA: see **Figure 3**.

considering the slight change in the CD spectra, it was suggested that the changes in gross secondary structure of OVA molecules by phosphorylation were mild. Increased surface hydrophobicity and enhanced SH−SS exchange reaction of OVA by phosphorylation were also observed. Thus, molten (partially unfolded) conformations formed by phosphorylation with pyrophosphate may play an important role during the subsequent heating for gelation, probably by promoting the formation of soluble aggregates through more extensive intermolecular interaction to induce firmer, more uniform gels.

To examine the stability of OVA against the heat-induced insolubility at pH 7.0, 0.1% solutions of N-OVA, DH-OVA, and PP-OVA dissolved in a 50 mM Tris-HCl buffer (pH 7.0) were heated at various temperatures (60−95 °C) for 10 min, and the soluble proteins were determined. As shown in **Figure 6**, the soluble proteins of N-OVA and DH-OVA (1 day and 5 days) decreased markedly as heating temperatures increased >70 °C and decreased ∼4% at 80 °C. In the case of PP-OVA-1d (dry-heated for 1 day), the soluble protein was >80% even when heated at 85 °C for 10 min, whereas the soluble proteins decreased rapidly when heated to temperatures >85 °C. The solubility of PP-OVA at pH 7.0 after heating was further improved by more phosphorylation through dry-heating for 5 days, even when heated at 95 °C for 10 min, and the soluble protein in solution remained >70%. The results suggested that the stability of OVA against heating at pH 7.0 was improved by phosphorylation. It has been reported that the electrostatic-repulsive force is important in helping to prevent the random aggregation of denatured OVA (*25*). In the present study, the improved stability of PP-OVA against heat-induced insolubility at pH 7.0 was considered to be due to the electrostatic-repulsive force of introduced phosphate groups.

Summarizing, in the present study, we have shown that the secondary structural change of OVA was mild, whereas the SH−SS exchange reaction was enhanced, and increased surface hydrophobicity and decreased Trp FI by phosphorylation were observed. Although the DSC thermograms of OVA showed a lowering of denaturation temperature by phosphorylation, the stability of OVA solution after heating at pH 7.0 was improved. The results indicated molten (partially unfolded) conformations of OVA formed by phosphorylation through dry-heating in the presence of pyrophosphate.

ABBREVIATIONS USED

OVA, ovalbumin; PP-OVA, OVA phosphorylated by dry-heating in the presence of pyrophosphate; CD, circular dichroism; DSC, differential scanning calorimetry; ANS, 1-anilino-8-naphthalenesulfonate; DH-OVA, OVA dry-heated in the absence of pyrophosphate; FI, fluorescence intensity; N-OVA, native OVA; P_i, inorganic phosphorus; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Trp, tryptophan.

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