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Improvement of Functional Properties of Egg White Protein through Phosphorylation by Dry-Heating in the Presence of Pyrophosphate

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Egg white protein (EWP) was phosphorylated by dry-heating in the presence of pyrophosphate at pH 3.0−7.0 and 85 °C for 1 and 5 days, and the functional properties of the phosphorylated EWP (PP-EWP) were investigated. The phosphorylation was accelerated with a decrease of pH from 7.0 to 3.0 and for heating times from 1 to 5 days. The phosphorus content of EWP increased ∼1.05% by dry-heating at pH 4.0 and 85 °C for 5 days in the presence of pyrophosphate, which was higher than that of casein. The electrophoretic mobility of EWP increased with an increase in the phosphorylation level. The surface hydrophobicity of EWP increased by phosphorylation. The heat stability, emulsifying properties, and digestibility of EWP were improved by phosphorylation. The calcium phosphate-solubilizing ability of EWP was enhanced by phosphorylation. A firmer and transparent heat-induced gel of PP-EWP was obtained, and the water-holding capacity of heat-induced PP-EWP gel was higher that that of the control. These results suggest that phosphorylation by dry-heating in the presence of pyrophosphate is a useful method for improving the functional properties of EWP.

KEYWORDS: Egg white protein; dry-heating; phosphorylation; surface hydrophobicity; heat stability; emulsifying properties; gelling properties; calcium phosphate-solubilizing ability

INTRODUCTION

Egg white protein (EWP) is exensively utilized as a functional food ingredient in the food industry, because it has nutritional and a wide range of functional properties. It is desirable for further industrial uses to improve their functional properties. Many attempts have been made to develop a rational molecular design using chemical and enzymatic modifications of proteins to improve their gelling, water-holding capacity (WHC), foaming, and emulsifying properties. Among these modifications, phosphorylation has been used to improve the functional properties of food proteins. For example, water solubility, emulsifying activity, foaming properties, and gel-forming properties of food proteins are improved by phosphorylation (*1*). Recently, the roles of phosphate groups in physiological (*2*−*4*) and immune (*5*−*7*) functions were reported. Accordingly, it is expected that the functional properties of food proteins are improved by phosphorylation. Chemical and enzymatic methods were developed for the phosphorylation of food proteins. Because violent reagents such as phosphorus oxychloride ($POCl_3$) and phosphorus pentoxide have been used in chemical phosphorylation (*1*, *8*), side reactions such as deterioration of amino acids and polymerization of proteins occur. Recently, Sitohy et al. (*9*) phosphorylated β-lactoglobulin with $POCl_3$ under mild conditions and succeeded in preparing proteins with few cross-linking molecules. However, chemically phosphorylated food proteins are not readily accepted by consumers due to the intense reaction and the difficulty of removal of remaining chemicals. Although enzymatic phosphorylation is the most desirable method for food proteins with respect to food safety (*10*, *11*), it brings in too few phosphate groups for the specificity of the substrate: the rate of phosphorylation was 2 mol of phosphorus/mol of acidic subunit of soybean protein (*10*). Such a low level phosphorylation is not enough to improve some functional properties of food proteins. Furthermore, this method does not seem to fit the needs of an industrial scale of production due to the high cost of enzymes. We have succeeded in introducing phosphate groups to ovalbumin and whey protein by conjugating them with glucose-6-phosphate (G6P) through the Maillard reaction (*12*, *13*). The prepared protein−G6P conjugates contained >1% phosphorus and showed excellent functional properties such as heat stability and emulsifying

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activity. The remaining problem of browning during the Maillard reaction is still unresolved.

Recently, Tarelli et al. (*14*) reported that saccharides and proteins that had hydroxyl groups could be phosphorylated when they were dried in the presence of phosphate. We have phosphorylated EWP by dry-heating in the presence of orthophosphate (*15*). Although some functional properties, such as heat stability and calcium phosphate-solubilizing ability of EWP have been somewhat improved by phosphorylation, the highest phosphorus content is 0.64%, which is insufficient for further improving some functional properties of EWP. To obtain the higher functional EWP, in this study, we phosphorylated EWP by dry-heating in the presence of pyrophosphate and described some functional properties of phosphorylated EWP (PP-EWP).

MATERIALS AND METHODS

Materials. EWP was prepared as follows: egg white, separated from infertile eggs purchased from Marui Agricultural Cooperative Association (Kagoshima, Japan), was homogenized, acidified to pH 5.5 with 1 N HCl, and then centrifuged. The supernatant obtained was diluted with an equal volume of water and dialyzed and then lyophilized. α-Chymotrypsin (type II) was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Anilino-8-naphthalenesulfonate (ANS) was purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Actinase E was purchased from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). All other reagents were of analytical grade.

Preparation of PP-EWP and Dry-Heated EWP (DH-EWP). EWP was dissolved at 20 g/L in 0.1 M sodium pyrophosphate buffer at pH 3.0−7.0 and the pH adjusted with 1 N HCl or NaOH; the solution was then 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-EWP, DH-EWP was prepared as follows: EWP was dissolved at a concentration of 20 g/L in deionized water and the pH adjusted to 3.0−7.0; the solution was then lyophilized and dry-heated under the same conditions as those of PP-EWP.

Determination of Phosphorus Content of PP-EWP. Protein samples were digested in perchloric acid. Phosphorus in the digest was regarded as the total phosphorus of PP-EWP. For the determination of inorganic phosphorus (P_i), 5 mL of 10% trichloroacetic acid was added to the same volume of 10 g/L PP-EWP solution, and the solution was centrifugated at 3000*g* for 20 min. The phosphorus in the supernatant was regarded as P_i. The phosphorus content was determined according to the method of Chen et al. (*16*). The amount of phosphorus bound to proteins was estimated by the difference between the total phosphorus and P_i content.

Measurement of Solubility of PP-EWP. Protein samples were dissolved at a concentration of 1 g/L 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. (*17*).

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE, 10% polyacrylamide, 8.5 cm × 7.5 cm × 1 mm) was performed in the absence of sodium dodecyl sulfate (SDS) using the buffer system of Laemmli (*18*), and then gel sheets were stained in Coomassie Blue G-250 for 1 h.

Evaluation of Surface Hydrophobicity. The surface hydrophobicity of EWP was evaluated by measuring the fluorescence intensity (FI) and initial slope (S_0) of EWP in the presence of ANS according to the method of Watanabe et al. (*19*). The EWP samples were dissolved in 20 mM phosphate buffer (pH 7.4), containing 0.1 mM EDTA to give 10 g/L EWP concentration, and then diluted with the same buffer for a series of three concentrations between 1 and 5 g/L. One milliliter of 0.3 mg/mL ANS solution in the same phosphate buffer was added to 1 mL of EWP solution as the fluorescence probe and then incubated at 25 °C for 1 h. FI was measured with a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) at an excitation wavelength of 390 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 fluorescence intensity versus protein concentration plot.

Measurement of Digestibility. Protease digestion was performed as follows: 20 mL of 1 g/L EWP solution in 50 mM Tris-HCl buffer (pH 8.0) was added to 100 μL of 10 g/L α-chymotrypsin or 100 μL of 10 g/L actinase E, respectively. Enzymatic reaction was carried out at 37 °C for 0−10 min at a protein/enzyme ratio of 20:1. After protease digestion, to stop the enzymatic reaction and remove native protein, 2 mL of protein solution taken from reaction solution was added to 2 mL of 10% aqueous trichloroacetic acid and then centrifugated at 3000*g* for 20 min. The amount of peptides and amino acids in the supernatant was measured according to the method of Lowry et al. (*17*). The extent of digestion was indicated as the digestion percentage of total protein.

Measurement of Emulsifying Properties. Emulsifying properties of EWP were measured according to the method of Pearce and Kinsella (*20*). To 3 mL of 1 g/L protein sample in 0.1 M phosphate buffer (pH 7.4) was added 1 mL of corn oil, after which the mixture was homogenized at 12000 rpm at 20 °C for 1 min with a PT10-35 Polytron homogenizer (Kinematic Ag, Switzerland). From the bottom of a test tube, 100 μL of emulsion was taken out at different times and diluted with 3 mL of 0.1% SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm. The emulsifying activity was determined from the absorbance measured immediately after emulsification. The emulsion stability was estimated by measuring the half-time of the turbidity measured immediately after emulsion formation.

Measurements of Heat Stability of EWP. Protein samples were dissolved at a concentration of 1 g/L 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. (*17*) to estimate the protein concentration of the solution. The heat stability described in this paper means the solubility of EWP after heat treatment. The visual inspection was observed by photography of EWP solution after heat treatment.

Preparation of Heat-Induced Gels for Measurement of Gelling Properties. The sample was dissolved in deionized water containing 50 mM NaCl to prepare a 100 g/L EWP solution. The EWP solution was degassed under vacuum for 10 min after the pH had been adjusted to 7.0 with 1 N NaOH; 150 μL of solution was transferred to each well of an eight-pore strip of a microplate, and the plate was affixed with a plastic film. Then, the microplate was heated in a bath at 80 °C for 40 min. The absorbance at 595 nm of solution was measured by using the Microplate Reader model 550 (Bio-Rad, Tokyo, Japan) before and after being heated (*21*). The EWP solution (12 mL) was transferred into a cylindrical casing tube made from polyvinylidene chloride (diameter = 13 mm, height = 100 mm) and then heated in a water bath at 80 °C for 40 min. The gel was immediately cooled to room temperature by immersion in tap water for 30 min and allowed to stand at room temperature for 1 h. The gel was sectioned at 6 mm thickness and used for measurement of mechanical properties at ambient temperature, which was carried out by the Texograph (Japan Food R&D Institute, Kyoto, Japan) equipped with a cylindrical plunger with a cross-sectional area of 0.125 cm^2. The plunger descended at a rate of 0.4 mm/s until the gel was ruptured, and then the direction was reversed to move upward at the same speed. Hardness, resiliency (elasticity), and compressibility (fractuability) of the gels were calculated from the force−deformation curves as follows (*22*).

$$\text{hardness} = \text{maximum force (gf/cm}^2\text{) at rupture in the compression curve (gf is gram-force)}$$

$$\text{resiliency} = (\text{area under the decompression curve/area under the compression curve}) \times 100\%$$

$$\text{compressibility} = (\text{depth of deformation at rupture/sample thickness}) \times 100\%$$

Measurement of WHC of Gels. The 100 g/L EWP solution prepared as described above was put into a cylindrical vinyl chloride

Table 1. Phosphorus Content and Solubility of EWP Dry-Heated at Various pH Values and 85 °C for 1 and 5 Days in the Presence of Pyrophosphate

pH	incubation time (days)	phosphorus content[a] (%)	solubility[b] (%)
–[c]	–	0.08	100
7.0	1	0.25	99.3 ± 2.1
7.0	5	0.33	98.5 ± 0.7
5.5	1	0.49	98.9 ± 0.9
5.5	5	0.60	96.2 ± 2.4
4.0	1	0.67	96.2 ± 2.1
4.0	5[d]	1.05	95.7 ± 2.5
3.0	1	0.89	95.8 ± 1.9
3.0	5	1.04	92.2 ± 3.4

[a] Data shown are the mean value of the two determinations, with a deviation of <1%. [b] The solubility of PP-EWP was measured at pH 7.0. Each value is the mean with its SD ($n = 3$). [c] The first sample is native EWP. [d] PP-EWP, which was dry-heated at 85 °C and pH 4.0 for 5 days in the presence of pyrophosphate, was used further for functional properties.

plastic casing (diameter = 13 mm, height = 150 mm) and then heated in a water bath at 80 °C for 40 min. The gels were immediately cooled to room temperature by immersion in tap water for 30 min and allowed to stand at room temperature for 1 h. WHC of gel was calculated from the formula

$$\text{WHC} = (W_1/W_0) \times 100\%$$

where W_0 is the initial gel weight and W_1 is the gel weight after being laid on five layers of filter paper (no. 2, diameter = 110 mm, Advantec) at ambient temperature (25 ± 1 °C) for 1 h.

Measurement of Solubilization of Calcium Phosphate by PP-EWP. The preparation of test solutions was done according to the procedures for artificial casein micelles (*23*). Two hundred microliters of 0.2 M potassium citrate, 200 μL of 0.2 M $CaCl_2$ and 240 μL of 0.2 M K_2HPO_4 were added to 2 mL of 4% protein solution followed by 100 μL of 0.2 M $CaCl_2$ and 50 μL of 0.2 M K_2HPO_4. The addition of 100 μL of 0.2 M $CaCl_2$ and 50 μL of 0.2 M K_2HPO_4 was repeated to give the concentrations of calcium and P_i of 30 and 22 mM, respectively. The interval set for addition was 15 min, and all additions were accompanied by stirring at pH 6.7. The volume was adjusted to 4 mL by measuring the weight of solution. The prepared solutions were allowed to stand for 20 h at 25 °C and then centrifuged at 3000*g* for 15 min. The calcium and P_i in the supernatant were then determined. Calcium was determined with a Hitachi Z-600 atomic absorption spectrophotometer (Hitachi Ltd., Tokyo, Japan) (*23*).

RESULTS

Phosphorylation and Solubility of PP-EWP. As shown in **Table 1**, the phosphorylation was accelerated with a decrease of pH from 7.0 to 3.0 and with an increase in the dry-heating period from 1 to 5 days. The phosphorus content of EWP dry-heated at 85 °C and pH 4.0 for 5 days was 1.05%, which is higher than that of casein (0.80%) (*15*). The solubility of PP-EWP was measured at pH 7.0. The solubility of EWP was almost unaffected by dry-heating at pH 5.5 and 7.0 for 1 day in the presence of pyrophosphate, which maintained a solubility of >98% (**Table 1**). Although the solubility of EWP decreased to 92.2% by dry-heating at pH 3.0 for 5 days in the presence of pyrophosphate, this value was much higher than the corresponding value of DH-EWP (74.2%) reported in a previous paper (*15*). Hence, the subsequent experiments on the functional properties were carried out using PP-EWP dry-heated at pH 4.0, because more insoluble protein was formed at pH 3.0 than at pH 4.0.

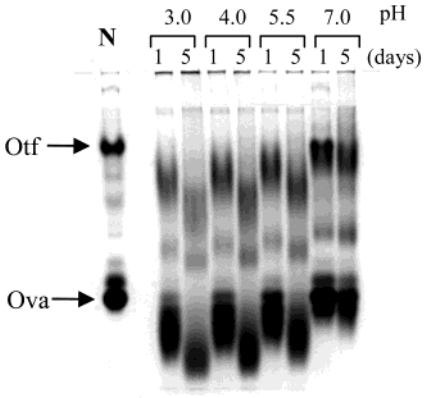

Figure 1. PAGE patterns of N- and PP-EWP, which were prepared by dry-heating at pH 3.0–7.0 and 85 °C for 1 and 5 days in the presence of pyrophosphate. PAGE (10% polyacrylamide gel without SDS) was performed at a constant current of 8 mA, and 15 μL of a 1 g/L sample solution was applied to each lane. N, native EWP; Ova, ovalbumin; Otf, ovotransferrin.

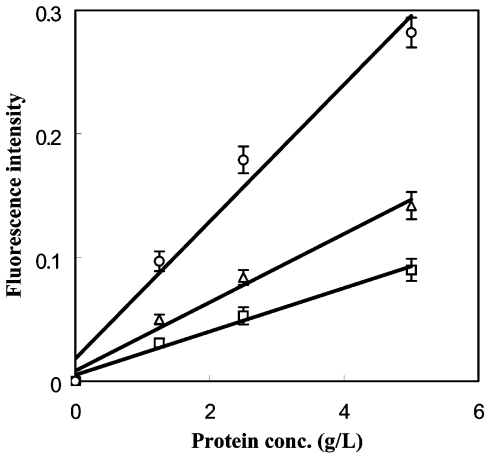

Figure 2. Fluorescence intensity of N- (□), DH- (△), and PP-EWP (○). DH- and PP-EWP were prepared by dry-heating at pH 4.0 and 85 °C for 5 days at pH 7.0. Each value is the mean with its SD ($n = 4$).

Figure 1 shows the native PAGE pattern of N- and PP-EWP. Compared with N-EWP, the mobility of proteins increased by dry-heating in the presence of pyrophosphate.

Properties of PP-EWP. The effect of phosphorylation on the surface hydrophobicity of EWP was evaluated by measuring the FI of EWP using the ANS binding method. **Figure 2** shows the FI of N-, DH-, and PP-EWP. The FI of EWP 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 111, which was 3.1-fold over that of N-EWP (35.2). These results suggest that phosphorylation of EWP through dry-heating in the presence of pyrophosphate caused the buried hydrophobic residues to become exposed.

Protease digestion of EWP was done by incubation with α-chymotrypsin or actinase E. As shown in **Figure 3**, the digestion velocity of native EWP with α-chymotrypsin or actinase E was slower than that of DH- or PP-EWP. The digestion velocities of PP-EWP, which were estimated as digestion of the first minute, were 10.58 and 14.10%/min for α-chymotrypsin and actinase E, respectively. These values were 7.4- and 3.7-fold over the values of N-EWP, respectively.

Functional Properties of PP-EWP. Figure 4 shows the emulsifying properties of N-, DH-, and PP-EWP. The value of

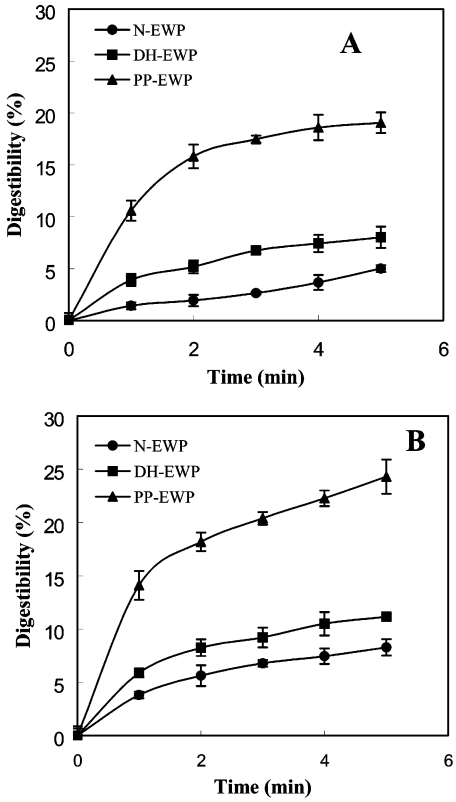

Figure 3. Time course of proteolytic digestion with α-chymotrypsin (**A**) or actinase-E (**B**) of N-, DH-, and PP-EWP. DH- and PP-EWP were prepared by dry-heating at pH 4.0 and 85 °C for 5 days. Each value is the mean with its SD ($n = 4$).

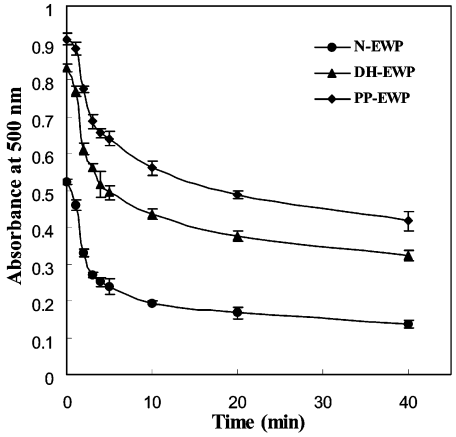

Figure 4. Emulsifying properties given as turbidity (A_{500nm}) of N-, DH-, and PP-EWP as a function of standing time after emulsification. The turbidity of the emulsion is plotted as the ordinate and standing time after emulsion formation as the abscissa. DH- and PP-EWP were prepared by dry-heating at pH 4.0 and 85 °C for 5 days. Each value is the mean with its SD ($n = 4$).

the ordinate at 0 time is relative emulsifying activity, and the half-life of initial turbidity reflects the stability of the emulsion. As presumed, the turbidity of emulsion from DH-EWP was much higher than that of N-EWP. Furthermore, the turbidity of emulsion from PP-EWP was higher than that from DH-EWP. The turbidity of PP-EWP at 500 nm was 0.91, which was 1.7-fold higher than that of N-EWP, and the half-time stability of emulsion turbidity of PP-EWP was 27.8 min, which was 8.4- and 2.2-fold longer than those of N- and DH-EWP, respectively.

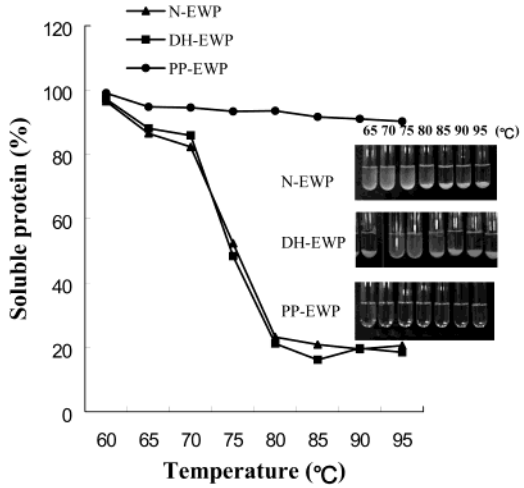

Figure 5. Heat stability of N-, DH-, and PP-EWP at various temperatures. The protein sample was 1 g/L in 50 mM Tris-HCl buffer (pH 7.0) and heated at 60–95 °C for 10 min. Data shown are the mean value of the two determinations, with a deviation of <1%. DH- and PP-EWP were prepared by dry-heating at pH 4.0 and 85 °C for 5 days.

To examine the heat stability of EWP, 1 g/L solutions of N-, DH-, and PP-EWP were heated at various temperatures (60−95 °C) for 10 min, and the soluble proteins were determined. As shown in **Figure 5**, the soluble proteins of N- and DH-EWP decreased markedly as heating temperatures increased >70 °C and decreased ∼20% at 80 °C. In the case of PP-EWP prepared by dry-heating at pH 4.0 and 85 °C for 5 days, >95% of proteins were soluble after heating at >70 °C for 10 min. These results show that PP-EWP has excellent heat stability. Visual inspection was done using photographs of EWP solution after heat treatment. As shown in the photograph in **Figure 5**, when heated at 70−75 °C, the N- and DH-EWP solutions became turbid and precipitated when the heating temperature was >80 °C. However, the PP-EWP solutions remained transparent when heated at all temperatures (65−95 °C).

The solubilization of calcium phosphate of DH- and PP-EWP was examined using the method of artificial casein micelles, where the final concentrations of calcium, P_i, and citrate were 30, 22, and 10 mM, respectively. The solubilized calcium and P_i were estimated from the difference between their soluble concentrations in the solution with and without protein. Without protein, the soluble calcium and P_i were 8.7 and 8.5 mM. In the presence of DH-EWP, the soluble calcium and P_i in the supernatant after centrifugation were 10.0 and 11.8 mM. These slight increases of soluble calcium and P_i may be due to the phosphate groups of ovalbumin or riboflavin-binding protein in EWP (*24*). The soluble calcium and P_i of supernatant in PP-EWP solution after centrifugation increased ∼27.3 and 21.2 mM, which were >90% of total calcium and P_i in solution **(Figure 6)**. In the presence of 2% PP-EWP, 18.6 mM calcium and 12.7 mM P_i were solubilized.

Properties of Gels. Table 2 shows the mechanical properties, turbidity, and WHC of heat-induced gels of N-, DH-, and PP-EWP. A rupture point of the N-EWP gel was not obtained during compression of the gel, because of its too soft texture. Dry-heating for 5 days in the absence of pyrophosphate increased the hardness, resiliency, and compressibility of gel of EWP, and dry-heating in the presence of pyrophosphate further increased these mechanical properties of gel. The hardness of the PP-EWP (5 days) gel was significantly lower than that of PP-EWP (1 day) gel; however, the resiliency, compressibility, and WHC were not different. Thus, 1 day of

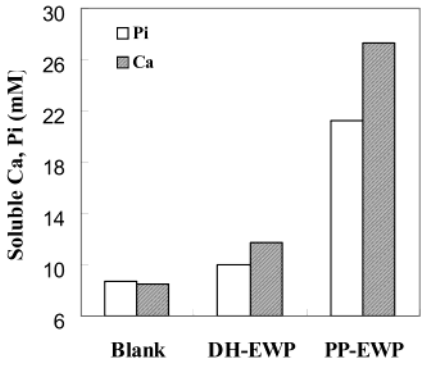

Figure 6. Calcium phosphate-solubilizing ability of DH- and PP-EWP. DH- and PP-EWP were prepared by dry-heating at pH 4.0 and 85 °C for 5 days. The test solution contained 20 g/L protein, 30 mM Ca, 22 mM P_i, and 10 mM citrate, and its pH was adjusted to 6.7 with 1 M KOH. Each column shows the mean value of the two determinations, with a deviation of <1%.

the dry-heating time is recommended when a heat-induced gel of PP-EWP is made. Furthermore, the turbidity of heat-induced gels from PP-EWP at 595 nm was much lower than those of gels from N- and DH-EWP. The gels of PP-EWP were transparent, whereas those of N- and DH-EWP were turbid by visual inspection**.** The WHC of gel from N-EWP was 84.8%, and this value was increased to 93.4% by dry-heating at pH 4.0 and 85 °C for 5 days in the absence of pyrophosphate. These values of EWP were further improved ~97.8 and 97.0% by dry-heating at 85 °C for 1 and 5 days in the presence of pyrophosphate, respectively.

DISCUSSION

We phosphorylated EWP by dry-heating in the presence of orthophosphate in a previous study (*15*), and some functional properties such as the heat stability and calcium phosphate-solubilizing ability of EWP were improved by phosphorylation. However, the introduced phosphorus content was still insufficient to improve some functional properties of EWP. In the present study, we phosphorylated EWP by dry-heating in the presence of pyrophosphate. The mobility of proteins increased by dry-heating in the presence of pyrophosphate. As described in a previous paper (*15*), almost no changes in the mobility were observed by dry-heating in the absence of phosphate. In the present study, the increased mobility of PP-EWP was observed. These results indicated that the negative charges of the phosphate groups were bound to the proteins and that a higher level of introduced phosphate groups caused greater mobility of EWP components. The phosphorus content of EWP by dry-heating in the presence of pyrophosphate was much higher than that of EWP dry-heated in the presence of orthophosphate. The phosphorus content of PP-EWP increased to1.05% by dry-heating at 85 °C and pH 4.0 for 5 days, which is higher than that of casein. This shows that phosphorylation occurred more efficiently by dry-heating in the presence of pyrophosphate than in the presence of orthophosphate.

Functional properties of some phosphorylated proteins have been studied and reviewed by Matheis and Whitaker (*1*). For example, Sung et al. reported that water solubility, emulsifying activity, foam expansion, and stability of soybean protein were improved by phosphorylation with $POCl_3$ (*25*). Calcium-binding ability of caseins was reported to be increased by phosphorylation with $POCl_3$ by Yoshikawa et al. (*26*). Woo and Richardson (*27*) reported that the emulsifying activity of phosphorylated β-lactoglobulin increased, and Heidelberger et al. (*28*) showed the loss of heat coagulation. On the other hand, Matheis et al. reported that the emulsifying activity of phosphorylated casein with $POCl_3$ was decreased (*29*). In the present study, there was only slight decrease in the solubility of PP-EWP dry-heated at pH 4.0 for 5 days in the presence of pyrophosphate. The emulsifying activity and stability of EWP were improved by dry-heating in the absence of pyrophosphate and were further improved by the introduced phosphate groups in EWP.

The repulsion of negative charges of introduced phosphate groups may make PP-EWP relatively more unfolded compared with DH-EWP, as confirmed in an increase of surface hydrophobicity. Thus, the dry-heating of EWP in the presence of pyrophosphate enhanced the hydrophobic residues, which thereby can occupy a large surface and subsequently improve the emulsifying properties of EWP. In the present study, ANS was used as a probe for measurement of the surface hydrophobicity of EWP. According to Nakai and Li-Chan (*30*), ANS binds to aromatic amino acid side chains from aromatic amino acid such as Phe, Trp, and Tyr, whereas CPA binds to aliphatic amino acid side chains from aliphatic amino acid such as Val, Leu, and Ile. Hydrophobicity measured with ANS shows a significant relationship with protein solubility, whereas hydrophobicity measured with *cis*-parinaric acid (CPA) does not. Although a marked increase in ANS surface hydrophobicity of PP-EWP was observed, the solublility of PP-EWP at pH 7.0 was higher. This may be due to the introduced negative charge of phosphate groups.

It has been reported that the electrostatic-repulsive force is important in helping to prevent the random aggregation of denatured ovalbumin (*31*). We have reported that the heat stability of EWP increased by dry-heating in the presence of orthophosphate but the soluble proteins in 1 g/L EWP solution decreased rapidly at >90 °C (*15*). In this paper, as shown in **Figure 5**, the PP-EWP dry-heated at pH 4.0 and 85 °C for 5 days shows excellent heat stability compared with N- and DH-EWP. This result shows that electrostatic repulsion of introduced phosphate groups prevents the aggregation of denatured proteins. It was suggested that the formation of a linear aggregate (transparent solution) or random aggregate (turbid solution) was

Table 2. Gelling Properties[a] of Heat-Induced Gel from N-, DH-, and PP-EWP

protein[b]	resiliency (%)	compressibility (%)	hardness (gf/cm^2)	turbidity of solution before/ gel after heating (A_{595nm})[c]	WHC[d] (%)
N-EWP	U[e]	U	U	0.18/1.98	84.8 ± 3.0
DH-EWP (5 days)	12.5 ± 2.1	58.0 ± 2.9	252 ± 20	0.15/1.99	93.4 ± 0.5
PP-EWP (1 day)	26.3 ± 7.7	78.4 ± 2.7	381 ± 23	0.06/0.13	97.8 ± 0.4
PP-EWP (5 days)	27.1 ± 6.7	76.5 ± 1.8	314 ± 4	0.10/0.14	97.0 ± 0.9

[a] Each value is the mean with its SD ($n = 3$). [b] N-EWP, native EWP; DH-EWP (5 days), EWP dry-heated at pH 4.0 and 85 °C for 5 days in the absence of pyrophosphate; PP-EWP (1 day) and PP-EWP (5 days), EWP dry-heated at pH 4.0 and 85 °C for 1 and 5 days, respectively, in the presence of pyrophosphate. [c] Data shown are the mean value of three determinations. [d] WHC, water-holding capacity. [e] U, unmeasurable.

controlled by the balance of hydrophobic interaction and electrostatic repulsion for the denaturation and aggregation of protein by heat treatment (*32*−*34*). The transparent solution obtained in this study may be due to the soluble linear aggregates of PP-EWP resulting from hydrophobic interaction and electrostatic-repulsive force between introduced phosphate groups.

Improvement of the gelling properties of EWP by dry-heating was reported by some researchers. Kato et al. (*35*, *36*) reported that a firm gel was obtained from DH-EWP. A similar result was obtained from ovalbumin by Matusdomi et al. (*34*). In our study, the textural properties including hardness, resiliency, and compressibility of EWP gel were improved by dry-heating in the presence of pyrophosphate. The heat-induced gel of PP-EWP was transparent. In the transparent PP-EWP gel, the improved textural properties may be due to the lesser aggregation of proteins than that of N- or DH-EWP. It is well-known that the gelling properties can be affected by the non-covalent cross-linkages such as hydrogen bonding and ionic and hydrophobic interactions and less frequently by covalent interactions (*37*). Furthermore, it has been suggested that the "molten" structure may strengthen these non-covalent cross-linkages (*38*). The marked improvement of WHC and transparency of PP-EWP gel may be explained by the existence of a uniform network in the gel.

We have reported that the calcium phosphate-solubilizing ability of EWP was enhanced by dry-heating in the presence of orthophosphate. More recently, Nakano et al. (*39*) reported that the calcium phosphate-solubilizing ability of phosphorylated starch and dextrin was introduced by dry-heating in the presence of phosphate. In this study, 18.6 mM calcium and 12.7 mM P_i were solubilized in the presence of 2% PP-EWP dry-heated at pH 4.0 and 85 °C for 5 days. These results show that the calcium phosphate-solubilizing ability of EWP was effectively enhanced by phosphorylation through dry-heating in the presence of pyrophosphate. Because the calcium phosphate-solubilizing ability of PP-EWP was confirmed, animal experiments are needed to evaluate the calcium bioavailability of PP-EWP.

The heat stability, emulsifying properties, gelling properties, and calcium phosphate-solubilizing ability of EWP were improved by dry-heating in the presence of pyrophosphate. Pyrophosphate, the chemical used in the phosphorylation reaction in the present study, is permitted as a food additive in Japan and is generally recognized as safe by the U.S. Food and Drug Administration (*40*). Furthermore, the dry-heating technology used in the food industry is generally recognized as safe. In this study, harmful components do not seem to be formed by dry-heating in the presence of pyrophosphate. From the above viewpoints, PP-EWP is therefore considered to be safe.

In conclusion, the results of the present study demonstrated that EWP could be effectively phosphorylated by dry-heating in the presence of pyrophosphate and that functional properties, including heat stability, emulsifying properties, gelling properties, and the calcium phosphate-solubility ability of EWP, were markedly improved by phosphorylation. Furthermore, potential physiological and immune functions of PP-EWP may be expected.

ABBREVIATIONS USED

ANS, 1-anilino-8-naphthalene sulfonate; DH-EWP, egg white protein dry-heated in the absence of pyrophosphate; EWP, egg white protein; FI, fluorescence intensity; G6P, glucose-6-phosphate; N-EWP, native EWP; PAGE, polyacrylamide gel electrophoresis; P_i, inorganic phosphorus; PP-EWP, EWP phosphorylated by dry-heating in the presence of pyrophosphate.

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