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Phosphorylation of Egg White Proteins by Dry-Heating in the Presence of Phosphate

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Food proteins were phosphorylated by heating in a dry state in the presence of phosphate. When casein, whey protein isolate (WPI), and egg white proteins (EWP), which were lyophilized from their solutions in a phosphate buffer, were dry-heated at various temperatures and pH levels for 1−5 days, EWP was more highly phosphorylated than casein and WPI. Phosphorylation of EWP was promoted with a decrease of pH from 7.0 to 3.0 when the incubation temperature was raised from 55 to 100 °C. The phosphorus content of EWP increased from 0.08 to 0.64% by dry-heating at pH 3.0 and 85 °C for 5 days in the presence of phosphate. The electrophoretic mobility of EWP increased with an increase in the phosphorylation level. The heat-induced polymerization of EWP by dry-heating was not affected by the presence of phosphate. Although the solubility of EWP decreased by dry-heating at pH 3.0−5.5, the phosphorylation depressed the insolubilization at low pH. The phosphate bonds in phosphorylated EWP (P-EWP) were stable at pH 2.0−10.0 and were more acid-labile and base-stable than phosphoesters of egg riboflavin-binding protein (RfBP). ^{31}P NMR spectral data suggested that besides phosphoesters, phosphodiester and polyphosphate bonds were introduced in P-EWP. Heat stability of EWP was improved, and calcium phosphate-solubilizing ability of EWP was enhanced by phosphorylation.

KEYWORDS: Egg white proteins; casein; egg riboflavin-binding protein; whey protein isolate; dry-heating; phosphorylation; functional property; heat stability; calcium phosphate-solubilizing ability

INTRODUCTION

Protein 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*). Milk caseins interact with calcium phosphate through their phosphate groups (*2*), and calcium and phosphate that are present in excess of their solubilities are solubilized (*3*). Egg white riboflavin-binding protein (RfBP), which constitutes ∼1% of egg white proteins (EWP), is a glycoprotein and comprises 219 amino acid residues containing 8 phosphoserine residues and also solubilizes calcium phosphate (*4*, *5*). Thus, phosphate groups can solubilize calcium phosphate and may enhance calcium absorption in the small intestine (*6*−*8*). Caseins have been shown to yield phosphopeptides upon digestion in the small intestine. These phosphopeptides also solubilize calcium phosphate and enhance calcium absorption in the small intestinum ileum. Recently, the roles of phosphate groups in physiological (*9*−*11*) and immune (*12*−*14*) 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 and phosphorus pentoxide have been used in chemical phosphorylation (*1*, *15*), side reactions such as the deterioration of amino acids and the polymerization of proteins occur. Chemically phosphorylated food proteins are not easily accepted by consumers due to its 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, it brings in too few phosphate groups for the specificity of the substrate (*16*, *17*). Such low-level phosphorylation is not enough to improve the functional properties of food proteins. Furthermore, this method does not seem to fit the need of industrial scale of production due to the high cost of enzymes. Aoki et al. (*18*, *19*) succeeded in introducing phosphate groups to ovalbumin and whey protein by conjugation with glucose-6-phosphate (G6P) through the Maillard reaction. The prepared proteins−G6P conjugates contained >1% phosphorus and showed excellent functional properties such as heat stability and emulsifying activity. The remaining problem of browning during the Maillard reaction is

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still unsolved. Recently, Tarelli et al. (*20*) reported that saccharides and proteins which had hydroxyl groups could be phosphorylated when they were dried in the presence of a phosphate buffer. This shows that if reaction conditions such as pH and temperature are adequate, introduction of the desired phosphate groups to improve functional properties may be possible.

The aims of the present study are to elucidate some factors of affecting phosphorylation of food proteins by dry-heating in the presence of phosphate and to characterize phosphorylated proteins. In this study, we describe phosphorylated food proteins by dry-heating in the presence of phosphate and describe some characteristic properties of phosphorylated EWP (P-EWP) and introduced phosphate bonds of it.

MATERIALS AND METHODS

Materials. EWP was prepared as follows: egg white, separated from infertile eggs, was purchased from Marui Agricultural Cooperative Association (Kagoshima, Japan), homogenized, acidified to pH 5.5 with 1 N HCl, and then centrifuged. The supernatant obtained was diluted with an equal volume of water, dialyzed, and then lyophilized.

RfBP was isolated from egg white according to the method of Hidaka and Matsuoka with a minor modification (*21*). Egg white was homogenized with a Waring blender, and the pH was adjusted to 5.5 with 1 N HCl. The precipitate thus formed was then removed by centrifugation. DEAE-Sephadex A-25 (20 g) preswollen with water was added to 1000 mL of the supernatant obtained, after which the suspension was gently stirred. Yellow DEAE-Sephadex A-25 was collected by decantation and washed three times with 0.05 M acetate buffer (pH 5.5) containing 0.1 M NaCl and once with 0.05 M acetate buffer (pH 4.0) containing 0.15 M NaCl. It was packed into a column. RfBP absorbed to the column was eluted with 0.05 M acetate buffer (pH 4.0) containing 0.5 M NaCl and then purified by gel filtration on a Sephacryl S-200 column (3.2 × 75 cm).

Casein was prepared from raw skim milk according to the acidic precipitation method.

Whey protein isolate (WPI) was purchased from Taiyo Kagaku Co., Ltd. (Yokkaichi, Japan).

α-Chymotrypsin (type II) and alkaline phosphatase (type I-S) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Preparation of Phosphorylated Proteins. Proteins were dissolved at a 2% concentration in 0.1 M sodium phosphate buffer at various pH values from 3.0 to 7.0 and then lyophilized. Lyophilized samples were incubated at various temperatures (55−100 °C) and heating periods (1 and 5 days). Dry-heated samples were dissolved and dialyzed to remove free phosphate for 3 days against distilled water and then lyophilized.

In comparison with P-EWP, dry-heated EWP (DH-EWP) was prepared as follows: EWP samples were dissolved at a 2% concentration in deionized water, and the pH of the solution was adjusted to 3.0−7.0, then lyophilized, and dry-heated under the same conditions as those of P-EWP.

Electrophoresis. Polyacrylamide gel electrophoresis (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 (*22*), and then gel sheets were stained in Coomassie Blue G-250 for 1 h.

High-Performance Liquid Chromatography (HPLC). Gel permeation HPLC was carried out at room temperature (25 °C) with a Hitachi UV detector L-7400 and D-2500 chromatograph (Hitachi Ltd., Tokyo, Japan), using a TSK-GEL G3000SW column (7.5 mm × 60 cm, Toso Ltd., Tokyo, Japan) fitted to a TSK guard column (7.5 mm × 7.5 cm). The elution buffer used was 0.1 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl, and 25 μL of sample solution (1 mg of protein/mL) was injected. The samples were eluted with the same buffer solution at a flow rate of 0.5 mL/min, and the elution profile was monitored by UV absorbance at 280 nm.

Acid and Base Stability of Phosphate Bonds. Proteins (1 mg/mL) in deionized water were adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 with 1 N NaOH or 1 N HCl or dissolved in 0.5 N HCl (pH 0.8−1.1) and 0.5 N NaOH (pH 12.2−13.2) and then incubated at 37 °C for 24 h. After incubation, total phosphorus and inorganic phosphorus (P_i) were determined.

Dephosphorylation of Proteins by Alkaline Phosphatase. Protein samples were dissolved at a protein concentration of 2 mg/mL in a 100 mM diethanolamine buffer, containing 0.5 mM $MgCl_2$ (pH 9.8). The sample solutions were incubated in a water bath at 37 °C, and then alkaline phosphatase was added in a protein/enzyme ratio of 20:1. The same protein samples were treated without alkaline phosphatase under the same conditions for comparison. The effect of proteolytic digestion of P-EWP on dephosphorylation was examinated by incubation with α-chymotrypsin for 2 h before the addition of alkaline phosphatase. After incubation, the total phosphorus and P_i were determined.

^{31}P Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{31}P NMR spectroscopy was carried out by a JNM-ECP 400-NMR spectrometer (JEOL Ltd., Tokyo, Japan) operating at 81 MHz and 20 °C. Phosphoric acid (85%) was used as an external standard. Proton-decoupled ^{31}P NMR spectra at pH 8.0 (adjusted with 1 N NaOH/D_2O solution) were obtained for DH-EWP and P-EWP prepared by dry-heating at 85 °C and pH 4.0 for 5 days. EWP samples were dissolved in a 0.7% urea and 0.7% SDS D_2O solution at a concentration of 30 mg/mL. The numbers of scans were 20000 for DH-EWP and 30000 for P-EWP. All spectra were obtained with an 80° tipping pulse and a 2-s repetition time.

Measurement of Solubility of EWP. Protein samples were dissolved in a 50 mM Tris-HCl buffer (pH 7.0) at a concentration of 0.1% and then centrifuged at 1000*g* for 20 min. The concentration of protein in the supernatant was determined by using the method of Lowry et al. (*23*). To detect the component of insoluble proteins, the precipitates of EWP dry-heated in the absence and presence of phosphate at 85 °C and pH 4.0 for 5 days were dissolved in a 2% SDS solution containing 5% 2-mercaptoethamol and analyzed by SDS-PAGE (15% polyacrylamide, 8.5 cm × 7.5 cm × 1 mm) according to the method of Laemmli (*22*).

Measurement of Heat Stability of EWP. Protein samples were dissolved at a protein concentration of 1 mg/mL of 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 1000*g* for 20 min. The soluble protein in the supernatant was measured to estimate the protein concentration of the solution. The heat stability described in this paper means the solubility of EWP after heat treatment.

Determination of Phosphorus Content of Phosphorylated Proteins. The protein samples were digested in the perchloric acid. Phosphorus in the digest and that in the trichloroacetic acid-soluble fraction were regarded as the total phosphorus and P_i, respectively. The phosphorus content was determined according to the method of Chen (*24*). The amount of bound phosphorus to proteins was estimated by subtracting the free phosphorus of supernatant from the total phosphorus.

Measurement of Solubilization of Calcium Phosphate by P-EWP. The preparation of test solutions was done according to the procedures for artificial casein micelles (*25*). To 2 mL of 4% protein solution were added 200 μL of 0.2 M potassium citrate, 200 μL of 0.2 M $CaCl_2$, and 240 μL of 0.2 M K_2HPO_4, 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 20 and 17 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 solutions. The prepared solutions were allowed to stand for 20 h at 25 °C and then centrifuged at 1000*g* for 15 min, and the calcium and P_i were determined in the supernatant. Calcium was determined by a Hitachi 208 atomic absorption spectrophotometer (Hitachi Ltd., Tokyo, Japan) using the filtrate of a mixture of solution containing 0.1 M HCl and 500 ppm of lanthanum.

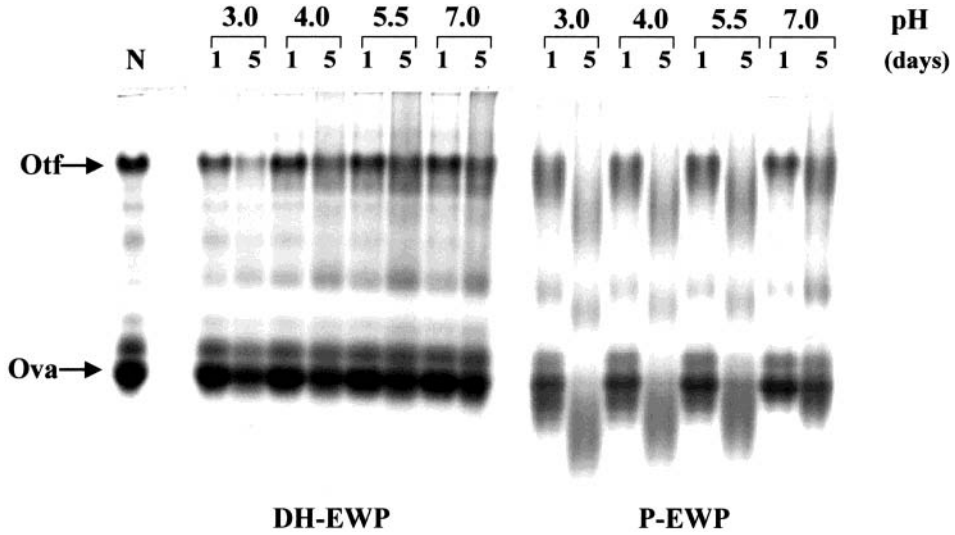

Figure 1. Native-PAGE patterns of native egg white protein (EWP), dry-heated EWP (DH-EWP), and phosphorylated EWP (P-EWP). DH-EWP and P-EWP were prepared by incubation at pH 3.0–7.0 and 85 °C for 1 and 5 days. Polyacrylamide gel electrophoresis (10% polyacrylamide, 8.5 cm × 7.5 cm × 1 mm) was performed at constant current of 8 mA, and 15 μL of a 0.1% sample solution was applied to each lane. N, native EWP; Ova, ovalbumin; Otf, ovotransferrin.

Table 1. Phosphorus Content of Proteins by Dry-Heating under Various Conditions in the Presence of Phosphate[a]

protein[b]	pH	incubation time (days)	incubation temp (°C)	P content (%)
WPI				0.05
	5.5	1	85	0.06
	5.5	5	85	0.14
CN				0.80
	5.5	1	85	0.80
	5.5	5	85	0.80
EWP				0.08
	7.0	1	85	0.10
	7.0	5	85	0.13
	5.5	1	85	0.24
	5.5	5	85	0.46
	4.0	1	85	0.28
	4.0	5	85	0.53
	3.0	1	85	0.35
	3.0	5	85	0.64
	5.5	1	55	0.11
	5.5	1	70	0.12
	5.5	1	100	0.31

[a] For further details see Materials and Methods. [b] WPI, whey protein isolate; CN, casein; EWP, egg white protein.

RESULTS

Phosphorylation. WPI, EWP, and casein were dissolved in a 0.1 M sodium phosphate buffer (pH 3.0−7.0) with a concentration of 2%, lyophilized, and then heated in the dry state at 85 °C for 1 and 5 days. **Table 1** shows the phosphorus content of phosphorylated proteins. The amount of phosphorylation was low in WPI, <0.1%. Casein contains 0.80% phosphorus, and almost no phosphorylation occurred in it. The phosphorylation level of EWP by dry-heating was higher than those of WPI and casein. EWP was phosphorylated under various conditions, including pH, temperature, and dry-heating time in the presence of phosphate. Phosphorylation was accelerated with a decrease of pH from 7.0 to 3.0 and with an increase in the temperature from 55 to 100 °C. The phosphorus contents of EWP incubated at 85 °C and pH 4.0 and 3.0 for 5 days were 0.53 and 0.64, respectively. These levels of phosphorylation were considered to be enough to improve the functional properties of EWP, because two phosphate groups of ovalbumin are responsible for its heat stability. Kitabatake et al. (*26*) reported that the ovalbumin with two phosphate groups, of which phosphorus content is estimated to be 0.14% as its molecular weight is 45000, was more heat-stable than the dephosphorylated ovalbumin. Accordingly, subsequent experiments were carried out using EWP.

Characteristics of Phosphorylated EWP. Figure 1 shows the polyacrylamide gel electrophoretic profiles of EWP dry-heated at 85 °C and various pH values from 3.0 to 7.0 for 1 and 5 days in the absence and presence of phosphate. In the absence of phosphate, there were almost no changes in the mobility of the components of EWP. However, the band of ovotransferrin became small in samples dry-heated for 5 days at pH 3.0. The band between ovalbumin and ovotransferrin appeared after dry-heating. This band was larger than in the samples of pH 5.5 and 7.0 and smaller than in that of pH 3.0 in the absence of phosphate. The mobility of proteins increased in the presence of phosphate. 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.

Protein polymerization during dry-heating was analyzed by gel permeation HPLC in the absence of SDS. **Figure 2** shows the typical elution profiles of EWP dry-heated at 85 °C and pH 5.5 in the absence and presence of phosphate. Peaks 1 and 2 were identified as ovotransferrin and ovalbumin, respectively. The peak area of ovalbumin decreased after dry-heating in the absence and presence of phosphate. The fraction eluted before peak 1 increased after dry-heating in the absence and presence of phosphate, suggesting that polymerized proteins formed during dry-heating, and there were almost no significant differences between EWP samples dry-heated in the presence and absence of phosphate. Similar results were obtained by HPLC analysis in the presence of SDS (data not shown). The polymerized proteins dissociated when the samples were reduced by 2-mercaptoethanol, suggesting that polymerization was caused mainly by intermolecular disulfide bonds. These results

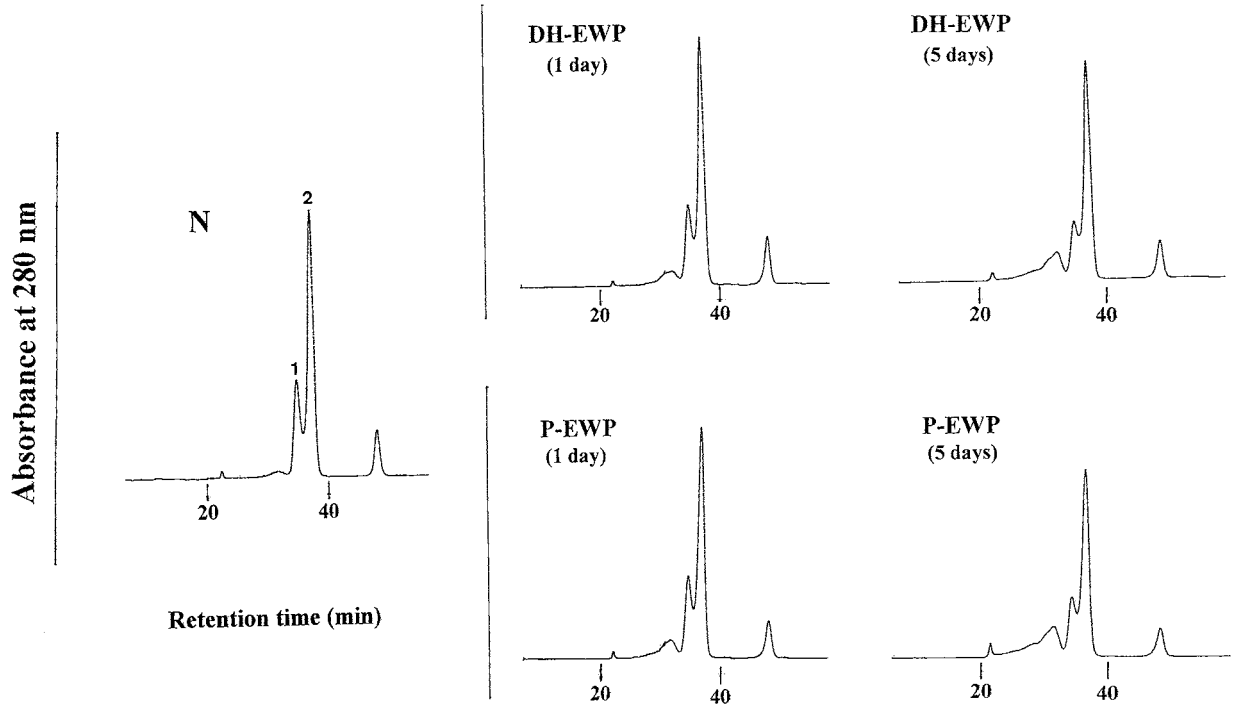

Figure 2. HPLC patterns of native EWP, DH-EWP, and P-EWP from a TSK-GELG3000SW column (7.5 mm × 60 cm). DH-EWP and P-EWP were incubated at pH 5.5 and 85 °C for 1 and 5 days. Elution buffer was 0.1 M phosphate buffer (pH 7.0) containing 0.3 M NaCl; flow rate = 0.5 mL/min. Peak 1, ovotransferrin; peak 2, ovalbumin; N, native EWP.

Table 2. Stability of the Phosphate Bonds in Phosphorylated EWP N(P-EWP) under Various pH Treatments[a]

	dephosphorylation (%)	
pH	P-EWP	RfBP
0.8–1.1[b]	43.1 ± 1.2	0.7 ± 0.2
2.0	1.4 ± 0.6	0.9 ± 0.3
3.0	0.9 ± 0.4	0.9 ± 0.3
4.0	1.6 ± 0.8	1.0 ± 0.5
5.0	0.9 ± 0.1	1.0 ± 0.1
6.0	0.6 ± 0.2	0.7 ± 0.4
7.0	0.8 ± 0.2	0.9 ± 0.1
8.0	1.0 ± 0.4	0.9 ± 0.4
9.0	2.0 ± 0.5	0.9 ± 0.3
10.0	1.2 ± 0.1	0.9 ± 0.2
12.2–13.2[c]	30.9 ± 1.8	90.0 ± 2.1

[a] For comparison, the stability of phosphate bonds in RfBP was examined under the same conditions as for P-EWP. Each value is the mean with its SD ($n = 3$). [b] Protein in 0.5 N HCl. [c] Protein in 0.5 N NaOH.

show that the polymerization of EWP caused by dry-heating was not affected by the presence of phosphate during dry-heating.

Characteristics of Phosphate Bonds. The stability of the phosphate bonds of P-EWP at various pH conditions was examined by incubation at 37 °C (**Table 2**). The dephosphorylation of P-EWP, which was incubated at pH 4.0 and 85 °C for 5 days, was <2% after incubation at 37 °C and pH 2−10 for 24 h, suggesting the phosphate bonds of P-EWP were stable at 37 °C and pH 2−10. Similar results were obtained in RfBP, which has eight phosphorylated serines. At 0.5 N HCl, the dephosphorylation levels of P-EWP and RfBP were 43.1 and 0.7%, respectively, suggesting the phosphate bonds of P-EWP were more acid-labile than those of RfBP. At 0.5 N NaOH, the dephosphorylation levels of P-EWP and RfBP were 30.9 and 90.0%, respectively, suggesting that the phosphate bonds of P-EWP were more base-stable than those of RfBP. Phosphate bound to the hydroxyl groups of protein serine and threonine residues is known to be base labile and acid stable (*27*). This was confirmed with RfBP (**Table 1**).

Enzymatic dephosphorylation was done to examine the characteristics of the phosphate bonds by alkaline phosphatase. The dephosphorylation of casein and RfBP under the same conditions was done for comparison. As shown in **Figure 3**, the dephosphorylation of P-EWP was 37.2% after incubation for 24 h with alkaline phosphatase. After digestion by α-chymotrypsin, the dephosphorylation of P-EWP increased to 61.3%. Almost no phosphate groups were released from P-EWP after incubation for 24 h without alkaline phosphatase. The dephosphorylation levels of casein and RfBP were 91.1 and 81.4%, respectively, under the same conditions, which were conspicuously higher than that of P-EWP. These results indicate that the phosphate groups in P-EWP could be due to phosphodiester or polyphosphates, whereas the native bonds are known to be phospho-monoester (*28*, *29*).

Figure 4 shows the ^{31}P NMR spectra of DH-EWP (A) and P-EWP (B) at pH 8.0. The chemical shifts are related to 85% H_3PO_4. Peaks near 4.7 ppm appeared in both proteins, which have been identified as phosphoester of phosphoserine, because the chemical shifts near 3.6−4.6 ppm at pH 8.0 have been representative of phosphoserine (*27*, *31*). The peaks at −6.6 and 5.3 ppm in P-EWP (**Figure 4B**), which were absent in DH-EWP (**Figure 4A**), were assigned to be related to phosphodiesters and most likely polyphosphates (*27*, *30*).

Functional Properties of P-EWP. The solubility is one of the most important characteristics of food proteins because it affects emulsifying and foaming properties. The solubility of EWP dry-heated in the absence and presence of phosphate was measured at pH 7.0. As shown in **Figure 5**, the solubility of EWP decreased after dry-heating in the absence of phosphate, especially at low pH, and the soluble protein of EWP dry-heated at 85 °C and pH 3.0 for 5 days was 74.2%. The decrease of solubility by dry-heating was depressed by phosphorylation. The

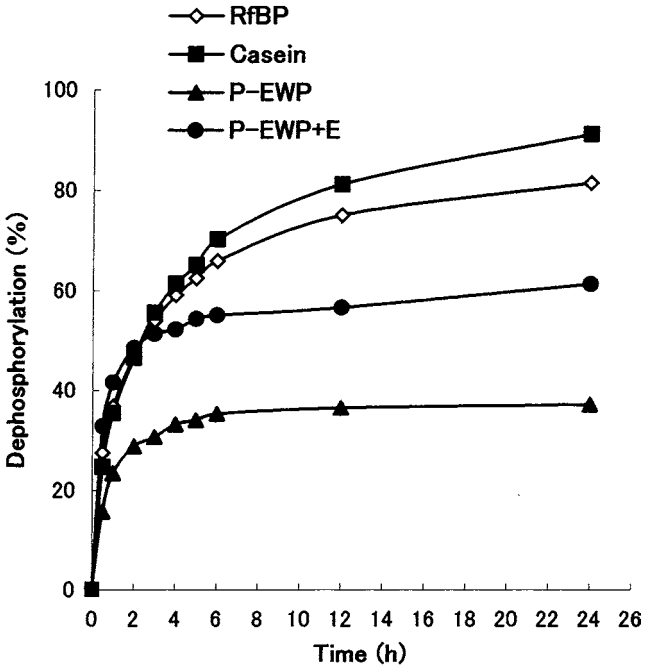

Figure 3. Dephosphorylation of various proteins by alkaline phosphatase in a protein/enzyme ratio of 20:1. P-EWP was prepared by incubation at pH 4.0 and 85 °C for 5 days. "P-EWP + E" means the P-EWP sample was dephosphorylated by alkaline phosphatase after proteolytic digestion by incubation with α-chymotrypsin for 2 h. Each line shows the mean value of the two determinations.

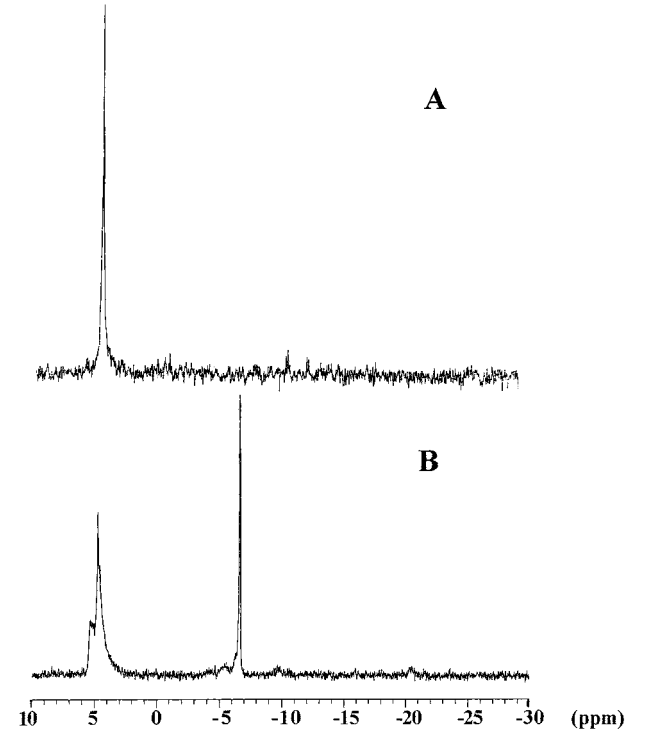

Figure 4. ^{31}P NMR spectrum at pH 8.0 and 20 °C of DH-EWP (A) and P-EWP (B), which were prepared by incubation at pH 4.0 and 85 °C for 5 days. H_3PO_4 (85%) was the external standard. Scan totals were 20000 for DH-EWP and 30000 for P-EWP. Resonance signals downfield to H_3PO_4 are positive.

solubility of P-EWP dry-heated at pH 4.0 and 85 °C for 5 days was >95%. The precipitates obtained by centrifugation of the EWP solutions were washed with water, lyophilized, and then dissolved in a 2% SDS solution containing 5% 2-mercaptoethamol and analyzed using SDS-PAGE. As shown in **Figure 6**, part of the proteins were aggregated into larger molecules and part of the polymerized proteins were difficult to introduce to the gel after dry-heating in both dry-heated EWPs. The bands of lysozyme in dry-heated samples, especially in P-EWP, were larger than that of native EWP, suggesting a part of the lysozyme became insoluble more readily by dry-heating. On the other hand, the ovalbumin and ovotransferrin bands became smaller in P-EWP than in DH-EWP. This showed that the insoluble ovalbumin and ovotransferrin were depressed by phosphorylation during dry-heating in the presence of phosphate.

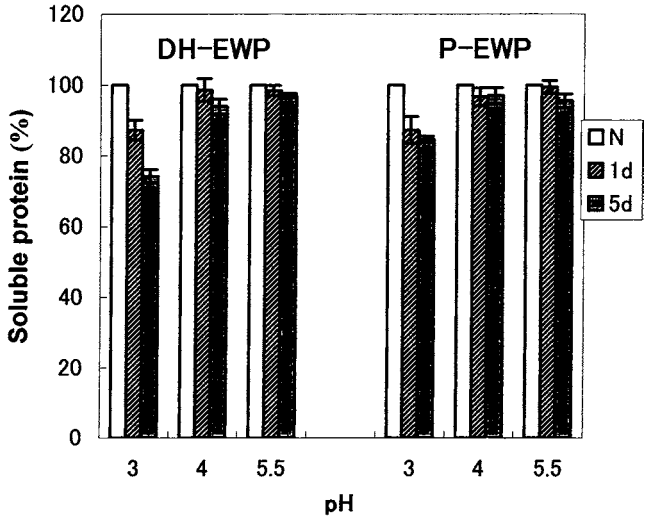

Figure 5. Solubility of native EWP, DH-EWP, and P-EWP. DH-EWP and P-EWP were prepared by incubation at pH 3.0–5.5 and 85 °C for 1 and 5 days. The protein samples were dissolved in the 50 mM Tris-HCl buffer (pH 7.0) at a 0.1% concentration. N, native EWP; 1d, dry-heating for 1 day; 5d, dry-heating for 5 days. Each value is the mean with its SD represented by a vertical bar ($n = 3$).

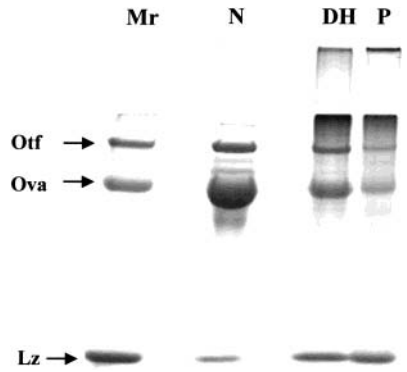

Figure 6. SDS-PAGE patterns of insoluble proteins in DH-EWP and P-EWP, which were prepared by incubation at pH 4.0 and 85 °C for 5 days. N, native EWP; Ova, ovalbumin; Otf, ovotransferrin; Lz, lysozyme.

To examine the heat stability of proteins, 0.1% solutions of native EWP, DH-EWP, and P-EWP were heated at various temperatures (60−95 °C) for 10 min, and the soluble proteins were determined. As shown in **Figure 7**, the soluble proteins of native EWP and DH-EWP decreased markedly as heating temperatures increased to >70 °C and decreased to ∼20% at 80 °C. In the case of P-EWP prepared by dry-heating at pH 4.0 and 85 °C for 5 days, 80.9% of proteins were soluble after heating at 85 °C for 10 min.

The solubilization of calcium phosphate of the P-EWP was examined using the method of artificial casein micelles, where the final concentrations of calcium, P_i, and citrate were 20, 17,

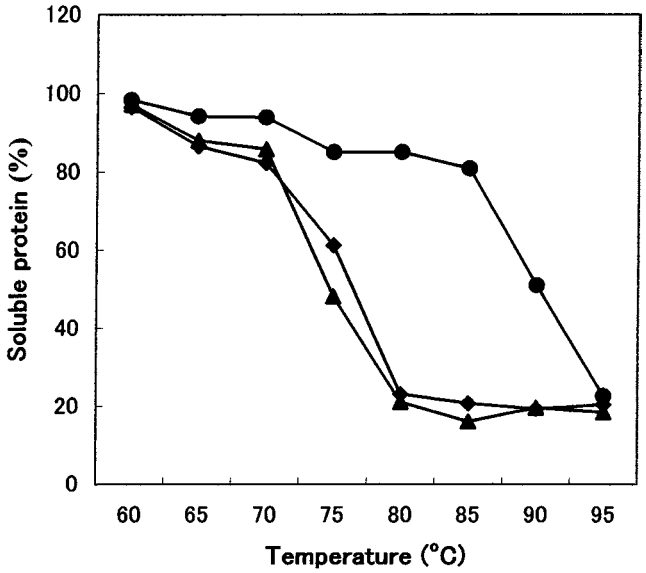

Figure 7. Heat stability of native EWP, DH-EWP, and P-EWP at various temperatures: (◆) native EWP; (▲) DH-EWP prepared by incubation at 85 °C and pH 4.0 for 5 days; (●) P-EWP prepared by incubation at 85 °C and pH 4.0 for 5 days. The protein sample was 0.1% in 50 mM Tris-HCl buffer (pH 7.0) and heated at 60–95 °C for 10 min. Each line shows the mean value of the two determinations.

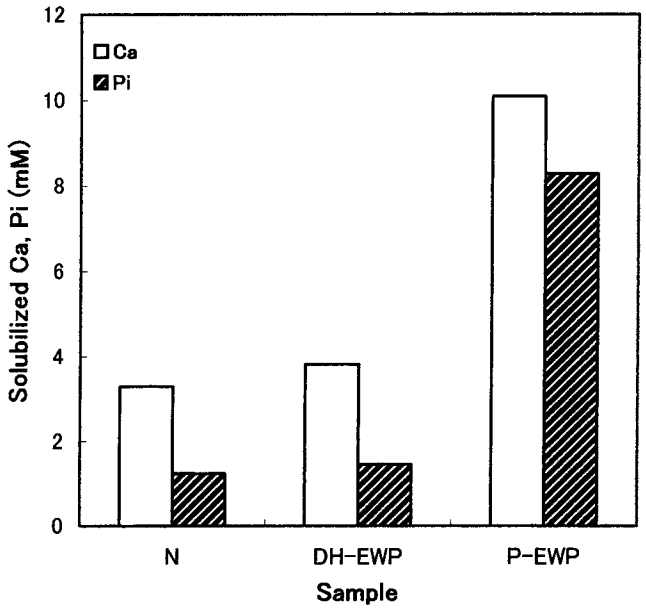

Figure 8. Calcium phosphate-solubilizing ability of native EWP, DH-EWP, and P-EWP. DH-EWP and P-EWP were prepared by incubation at 85 °C and pH 4.0 for 5 days. The test solution contained 2% protein, 20 mM Ca, 17 mM P_i, and 10 mM citrate, and its pH was adjusted to 6.7 with 1 M KOH. Each column shows the mean value of the two determinations.

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. Although EWP had only slight calcium phosphate-solubilizing ability, it was enhanced by phosphorylation. In the presence of 2% protein prepared by incubation at pH 4.0 and 85 °C for 5 days, P-EWP solubilized 10.1 mM calcium and 8.3 mM P_i (**Figure 8**).

DISCUSSION

Dry phosphorylation of starch with phosphate under heat and vacuum has been reported by Sitohy et al. (*32*). This shows saccharides can be phosphorylated by dry-heating in the presence of phosphate. In the present study, EWP contains more saccharides than do WPI and casein. It is reasonable that higher phosphorylation occurs in EWP. However, the results of ^{31}P NMR indicate that phosphate bonds, which were different from phosphoester bonds, were introduced in EWP by dry-heating in the presence of phosphate. The peaks at −6.6 and 5.3 ppm in P-EWP (**Figure 4B**), which were absent in DH-EWP (**Figure 4A**), have been assigned to be related to phosphodiesters and most likely polyphosphates (*27*, *30*). Furthermore, the dephosphorylation patterns of P-EWP by alkaline phosphatase, acid, or base treatments also indicate that the introduced phosphate bonds are different from phosphoesters of casein or RfBP and could be due to phosphodiester or polyphosphate. This was further confirmed from the result of ^{31}P NMR. The peak near 5.2 ppm has been reported to be sugar phosphate by Wahlgren et al. (*33*). In our study, the peak at 5.3 ppm is also assumed to be sugar phosphate, because the major proteins of EWP (ovalbumin and ovmucoid) are glycoproteins. Thus, the sugar moiety would be expected to be phosphorylated (*20*) under the conditions used in this study. On the other hand, nitrogen-bound phosphate bonds that appear near −2.4 through −3.2 ppm (*27*, *33*) were not detected in P-EWP.

After proteolytic digestion, the dephosphorylation of EWP increased. This indicates that part of the phosphate groups are located in the interior of EWP. Further study is needed to identify the amino acid residues to which the phosphate groups are bonded.

The phosphorylation process in the dry state may be explained in simple terms by the following reversible reaction:

$$◆-OH + H_3PO_4 \rightleftharpoons ◆-O-H_2PO_3 + H_2O$$

Here, ◆ represents a protein or sugar. It was promoted with an increase of dry-heated temperature from 55 to 100 °C. Removal of water from this system will promote the forward reaction, because of Le Chatelier's principle, and, hence, drying will promote the formation of esters. A small amount of residual water, however, appears to facilitate the reaction, and this is similar to the formation of Schiff bases in dry mixtures of proteins and reducing sugars for which there is reported to be an optimum moisture content for maximum reaction rate (*34*). The role of water in this reaction could be physical, providing a degree of mobility within the solid matrix that may facilitate any possible reactions. Water may also, in the present case, be involved in the reaction itself. Phosphorylation was also enhanced by the decrease of pH from 7.0 to 3.0. At pH 5.5 and lower, the main anionic phosphate species present is the monoanion, $H_2PO_4^-$. By increasing the amount of this species, it is reasonable that phosphoester formation between hydroxyl and $H_2PO_4^-$ is more favorable than that between hydroxyl and HPO_4^{2-}. Therefore, the decrease of pH from 7.0 to 3.0 could enhance phosphorylation.

The functional properties of phosphorylated food proteins have been studied by Matheis and Whitaker (*1*). Woo and Richardson (*35*) reported that phosphorylated β-lactoglobulin increased emulsifying activity. The improved emulsifying and foaming properties and digestibility of yeast protein by chemical phosphorylation with phosphorus oxychloride were reported by Huang et al. (*36*). Using the enzymatic phosphorylation method, a phosphorylated soy protein isolate, which has good solubility and emulsifying activity, was obtained by Campbell et al. (*17*). The calcium phosphate-solubilizing ability of phosphorylated β-lacoglobulin-G6P was reported by Aoki et al. (*19*). In our study, through phosphorylation of EWP by dry-heating in the

presence of phosphate, heat stability was improved. The calcium phosphate-solubilized ability of EWP also was improved by phosphorylation, which may be expected to increase the absorption of calcium.

Kato et al. (*37*) reported that the amino acids of EWP, which were dry-heated at 80 °C for 5 days, had almost no damage. In this study, there was almost no browning during dry-heating. Although part of lysozyme became insoluble, the insoluble proteins of ovalbumin and ovotransferrin, formed during dry-heating, were depressed by dry-heating in the presence of phosphate (**Figure 6**). Furthermore, phosphate, the chemical used in the phosphorylation reaction in this study, is allowed as an additive in foods. From this viewpoint, the chemical used in this study is considered to be safe. Additionally, phosphate is cheap and can be utilized on an industrial scale.

Recently, the roles of phosphate groups have been reported to include physiological and immune functions. Hata et al. (*13*) identified bovine β-casein (1−25) and α-casein (59−79) as having phosphoserine-rich regions acting as immunomodulating factors in trypsin digests of bovine caseins. Otani et al. (*15*) have observed that the oral administration of commercially available casein phosphopeptide preparation increases intestinal total and antigen-specific IgA levels in mice and piglets. The physiological role of phosphate groups in phosphopolysaccharide has been reported by Kitazawa et al. (*10*−*12*). Thus, the physiological and immune function of phosphorylated proteins can be expected.

The results of the present study demonstrated that phosphate groups could be introduced to proteins by dry-heating in the presence of phosphate and that functional properties, such as heat stability and calcium phosphate-solubilizing ability of EWP, were improved by phosphorylation.

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