



Improvement of Functional Properties of Egg White Protein through Glycation and Phosphorylation by Dry-heating*

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ABSTRACT : Egg white protein (EWP) was glycated with maltopentaose (MP) through the Maillard reaction and subsequently phosphorylated by 85°C dry-heating at pH 4.0 for 1 d in the presence of pyrophosphate. The functional properties of glycated, phosphorylated EWP were compared with those of native EWP and with EWP which was phosphorylated by dry-heating in the presence of pyrophosphate under the same conditions. The phosphorus content of EWP was increased to ~0.60% by phosphorylation, and to ~0.74% by glycation with MP and subsequent phosphorylation. The electrophoretic mobility of EWP increased through phosphorylation. The stability of EWP against heat-induced insolubility at pH 7.0 was considerably improved by phosphorylation alone and further by phosphorylation after glycation. The anti-ovalbumin antibody response was reduced significantly by glycation and phosphorylation, and further reduced by phosphorylation after glycation. The anti-ovomucoid antibody response was reduced significantly by glycation, phosphorylation and phosphorylation after glycation. The calcium phosphate-solubilizing ability of EWP was enhanced by both phosphorylation methods. (**Key Words :** Egg White Protein, Phosphorylation, Dry-heating, Maillard Reaction, Functional Property)

INTRODUCTION

Egg white protein (EWP) is extensively utilized as a functional food ingredient in the food industry, because it has nutritional and a wide range of functional properties. It is desirable to improve their functional properties for further industrial uses. 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, foaming, and emulsifying properties. Among these modifications, phosphorylation has been proven to be a useful method for improving the functional properties of food proteins (Matheis and Whitaker, 1984). Over the past few decades, several phosphorylation

methods have been reported by some researchers (Seguro and Motoki, 1989; Aoki et al., 1994, 1997; Kato et al., 1995; Sitohy et al., 1995; Vojdani and Whitaker, 1996). However, these phosphorylation methods have posed some problems (Li et al., 2003, 2004), making them very difficult to put to practical use. Li et al. (2003) phosphorylated EWP by dry-heating in the presence of phosphate, significantly improving the heat stability, emulsifying properties, and gelling properties of EWP. Furthermore, the calcium phosphate-solubilizing ability of EWP was enhanced by phosphorylation. However, whey protein isolate showed a lower phosphorylation level than EWP by dry-heating under the same conditions, presumably due to the lower sugar content of whey protein isolate (Li et al., 2003). We then attempted to prepare phosphorylated whey protein isolate by glycation with maltopentaose (MP) through the Maillard reaction and subsequent dry-heating in the presence of pyrophosphate, with the result that some functional properties were improved by phosphorylation after glycation (Li et al., 2005a). Further study revealed that the antigenicity of β -lactoglobulin and bovine serum albumin, major allergens in whey protein isolate, were significantly reduced by glycation with MP or glycation and phosphorylation, respectively (Enomoto et al., 2007, 2008).

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In the present study, we phosphorylated EWP by dry-heating in the presence of pyrophosphate after glycation with MP, and the functional properties of glycated and subsequently phosphorylated EWP were compared with those of native EWP and phosphorylated EWP by dry-heating in the presence of pyrophosphate. Ovalbumin (OVA) and ovomucoid (OVM) are shown to be major allergens in EWP (Mine and Rupa, 2004). In particular, OVM is the dominant allergen in EWP and is the cause of most allergic reactions in children (Mine and Rupa, 2004). Thus, the effect of both phosphorylation methods on the antigenicity of OVA and OVM in EWP are also examined.

MATERIALS AND METHODS

Materials

Native EWP (N-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. Bovine serum albumin (essentially globulin free), MP were purchased from Nacalai Tesuque Co., Ltd. (Kyoto, Japan). All other reagents were of analytical grade.

Preparation of phosphorylated EWP

EWP was dissolved in 0.1 M sodium pyrophosphate buffer at pH 4.0. The lyophilized sample was incubated at 85°C for 1 d and then dialyzed against Milli-Q water for 3 d, after which the solution was then lyophilized (PP-EWP).

EWP and MP (1:0.3 wt/wt) were dissolved in Milli-Q water at a protein concentration of 20 g/L, and the solution pH was adjusted to 8.0 with 1 N NaOH, followed by lyophilization. The dried sample was kept at 50°C and 65% relative humidity (RH) for 3 d using a saturated KI solution in a desiccator according to the method given in a previous paper (Aoki et al., 2001) and was then dissolved in 0.1 M sodium pyrophosphate buffer at pH 4.0. The lyophilized sample was incubated at 85°C for 1 d and then dialyzed against Milli-Q water for 3 d, after which the solution was then lyophilized (PP-MP-EWP).

For the preparation of EWP conjugated with MP, EWP and MP (1:0.3 wt/wt) were dissolved in Milli-Q water at a protein concentration of 20 g/L, and the pH of the solution was adjusted to 8.0 with 1 N NaOH, followed by lyophilization (MP-EWP). The dried sample was kept at 50°C and 65% RH for 3 d using a saturated KI solution in a desiccator and then dialyzed against Milli-Q water for 3 d, after which the solution was then lyophilized. For the preparation of dry-heated EWP, EWP was dissolved in Milli-Q water at a concentration of 20 g/L, and the pH of the solution adjusted to 4.0 with 1 N HCl, followed by

lyophilization. The lyophilized sample was incubated at 85°C for 1 d and then dialyzed against Milli-Q water for 3 d, after which the solution was then lyophilized (DH-EWP).

These dried samples were stored at -20°C before use of the subsequent experiment.

Determination of sugar content

The total sugar contents of protein samples were determined according to the phenol-sulfuric acid method (Dubois et al., 1956). For the determination of free sugar, 2 ml of a 2 g/L sample solution was ultrafiltered through Centriscalt I (Sartorius AG-W-3400, Goettingen, Germany; molecular mass cut off = 10,000). The sugar content in the ultrafiltrate was regarded as free sugar. The sugar bound to EWP was estimated by the difference between the total and free sugar contents.

Determination of phosphorus content

Protein samples were digested in perchloric acid. Phosphorus in the digest was regarded as the total phosphorus of protein. For the determination of inorganic phosphorus (Pi), 2 ml of 2 g/L sample solution was ultrafiltered through Centriscalt I (Sartorius AG-W-3400, molecular mass cut off = 10,000). The phosphorus content in the ultrafiltrate was regarded as Pi. The P content was determined using the method of Chen et al. (1956). The amount of phosphorus bound to proteins was estimated by the difference between the total phosphorus and Pi content.

Measurement of solubility and stability against heat-induced insolubility

Protein samples were dissolved at a protein concentration of 1 g/L in 50 mM Tris-HCl buffer (pH 7.0). For the measurement of solubility, the protein solution was centrifuged at 1,000 g for 15 min. For the measurement of stability against heat-induced insolubility, the sample solutions (1 ml) were placed in small test tubes with aluminum foil stoppers and heated in a water bath at 60-95°C for 10 min. Aggregates were precipitated by centrifugation at 1,000 g for 30 min.

Soluble protein in the supernatant was measured to estimate the protein concentration of the solution by the method of Lowry et al. (1951).

Electrophoresis

Native polyacrylamide gel electrophoresis (native PAGE) was performed using 10.0% gels in the absence of sodium dodecyl sulfate (SDS) and SDS-PAGE using 10.0% polyacrylamide gels under reducing conditions in the presence of 2-mercaptoethanol (2-ME) according to the method of Laemmli (1970). The gels were stained in Coomassie Brilliant Blue R-250 for 1 h.

Table 1. Some characteristics of egg white protein (EWP) evaluated

Sample ¹	Sugar content ² (%)	P content ² (%)	Solubility ² (%)
N-EWP	3.2±0.4	0.09±0.01	98.2±1.6
DH-EWP	3.4±0.4	0.08±0.01	95.6±2.3
MP-EWP	5.9±0.2	0.08±0.01	97.4±1.0
PP-EWP	3.2±0.3	0.60±0.01	96.2±2.1
PP-MP-EWP	8.3±0.4	0.73±0.06	95.9±0.8

¹ N-EWP = Native EWP; DH-EWP = EWP dry-heated at pH 4.0 and 85°C for 1 d; MP-EWP = EWP conjugated with MP by incubation at 50°C (65% RH) for 3 d; PP-EWP = EWP dry-heated at pH 4.0 and 85°C for 1 d in the presence of pyrophosphate. PP-MP-EWP = MP-EWP dry-heated at pH 4.0 and 85°C for 1 d in the presence of MP and pyrophosphate.

² Each value is the mean±SD (n = 3).

Enzyme-linked immunosorbent assay (ELISA)

A noncompetitive ELISA was carried out according to the previous paper (Enomoto et al., 2007). EWP samples dissolved in PBS (0.11 M phosphate buffer, pH 7.1, containing 0.04 M NaCl and 0.02% NaN₃) at a protein concentration of 0.1 g/L (100 µl) were added to the wells of a polystyrene microtitration plate (Maxisorp; Nunc A/S, Roskilde, Denmark), and the plate was incubated at 4°C overnight to coat the wells with each antigen. After the removal of the solution, each well was washed four times with 200 µl of PBS-Tween (PBS containing 0.5 g/L Tween 20). A 20 g/L bovine serum albumin/PBS solution (120 µl) was added to each well, and the plate was incubated at 25°C for 2 h and then washed four times. One hundred microliters of the anti-OVA (CH-002, Cosmo Bio Co., Ltd., Tokyo, Japan) or anti-OVM (CH-006, Cosmo Bio Co., Ltd., Tokyo, Japan) monoclonal antibody (IgG1) dissolved in PBS was added to each well, and the plate was incubated at 25°C for 2 h. After four washings, 100 µl of alkaline phosphatase-labeled goat anti-mouse immunoglobulin G₁ (1070-09, SouthernBiotech, Birmingham, USA) diluted with PBS-Tween was added to each well. The plate was incubated at 25°C for 2 h, and the wells were then washed four times. One hundred microliters of 1 g/L sodium *p*-nitrophenyl phosphate disodium/diethanolamine hydrochloride buffer (pH 9.8) was added to each well, and the plate was incubated at 25°C for 30 min. After the addition of 5 M sodium hydroxide solution (20 µl) to each well to stop the reaction, the absorbance at 405 nm was measured using a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA).

Measurement of solubilization of calcium phosphate

The preparation of test solutions was conducted according to the procedures for artificial casein micelles (Aoki, 1989). Forty microliters of 1.0 M potassium citrate, 200 µl of 0.2 M CaCl₂, and 240 µl of 0.2 M K₂HPO₄ were added to 2 ml of 4% protein solution, followed by the

addition of 200 µl of 0.2 M CaCl₂ and 100 µl of 0.2 M K₂HPO₄. The addition of 200 µl of 0.2 M CaCl₂ and 100 µl of 0.2 M K₂HPO₄ was repeated to yield calcium and Pi concentrations of 30 and 22 mM, respectively. The interval set for the addition was 15 min, and all additions were accompanied by stirring at pH 6.7. The volume was adjusted to 4 ml (2% protein concentration) by measuring the weight of the solutions. The prepared solutions were allowed to stand for 20 h at 25°C and then centrifuged at 1,000 g for 15 min. The calcium and P_i in the supernatant were then determined (the former by using a Hitachi Z-600 atomic absorption spectrophotometer, Hitachi Ltd., Tokyo, Japan).

Statistical analysis

The data are expressed as mean values with standard deviation (SD). Significant differences between mean values are determined by Student's *t*-test at the 5% significance level.

RESULTS AND DISCUSSION

Characteristics of phosphorylated EWP

EWP was phosphorylated by 85°C dry-heating at pH 4.0 for 1 d in the presence of pyrophosphate (PP-EWP), or conjugated with MP at pH 8.0 and 50°C (65% RH) for 3 d through the Maillard reaction (MP-EWP), and MP-EWP was then phosphorylated by 85°C dry-heating at pH 4.0 for 1 d in the presence of MP and pyrophosphate (PP-MP-EWP). Table 1 shows some characteristics of the various EWP samples. Although the sugar content of EWP was 3.2%, after incubation with MP at 50°C (65% RH) for 3 d, the sugar content of EWP increased to 5.9% and then further still to 8.3% by dry-heating at pH 4.0 and 85°C for 1 d in the presence of MP and pyrophosphate. This suggested that glycation occurred efficiently on EWP. The phosphorous content of EWP was 0.09%, whereas that of EWP increased to 0.60% by dry-heating for 1 d in the presence of pyrophosphate. Furthermore, the phosphorous content of MP-EWP increased to 0.74% at the same conditions. The saccharides or sugar chains in protein are phosphorylated by dry-heating in the presence of phosphate (Tarelli and Wheeler, 1994; Nakano et al., 2003), suggesting that introduction of sufficient sugar may result in more efficient phosphorylation of EWP by dry-heating in the presence of pyrophosphate.

The solubility of food protein is an important property for its application in food processing. The solubility of EWP samples was measured at pH 7.0. Almost no effect of the Maillard reaction or phosphorylation on the solubility of EWP was observed; the solubility of PP-EWP and PP-MP-EWP was 96.2% and 95.8%, respectively.

Native PAGE was performed to elucidate the changes of

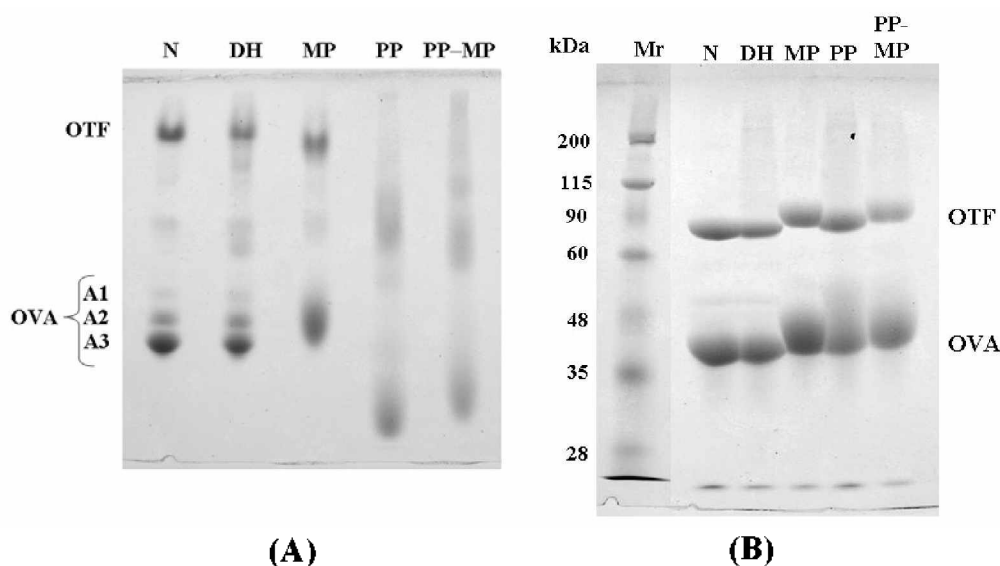


Figure 1. Electrophoretic patterns of native (N), dry-heated (DH), maltopentaose-conjugated (MP), phosphorylated (PP), and phosphorylated and maltopentaose-conjugated (PP-MP) egg white protein (EWP): (A) Native-PAGE (10% polyacrylamide gel in the absence of SDS), (B) SDS-PAGE (10% polyacrylamide gel in the presence of 1.7% SDS) with 5% of 2-ME. Lane Mr = marker protein.

charge in protein by phosphorylation. Figure 1A shows the native PAGE pattern of EWP samples. There were almost no changes in the mobility of N- and DH-EWP components, whereas glycation with MP increased the mobility of ovotransferrin (OTf) and decreased the mobility of OVA. As glycation modifies basic amino acid side chains, it induces a slight loss of basicity and, consequently, a moderate acidification of the OVA and OTf in EWP. However, the mobility of OVA in MP-EWP decreased, which might be caused by the introduction of MP to the OVA and the subsequent increase of their molecular mass (Li et al., 2005a). On the other hand, the mobility of PP- and PP-MP-EWP components increased. These results indicated that the negatively charged phosphate group on EWP components produced mobility.

To assess the formation of aggregates, we performed SDS-PAGE in the presence of 2-ME. As shown in Figure 1B, the monomer of OTf and OVA were clearly observed in N-EWP. When EWP was dry-heated for 1 d in the absence of MP and pyrophosphate, the intensities of the bands of aggregates slightly increased and relative concentration of those monomers decreased, but almost no changes in the mobility of those monomers were observed. However, the mobility of those monomers decreased by glycation. This observation indicated that the molecular mass of EWP components increased by conjugation with MP, which might explain why the mobility of OVA in EWP decreased by conjugation with MP in the native PAGE (Figure 1A). Some of the aggregates remained undissociated, indicating that not only disulfide bonds but also other types of bonds were formed by dry-heating. Although covalent bonds other than the disulfide bonds formed in proteins by dry-heating

have been discussed by some researchers (Kato et al., 1989; Watanabe et al., 1999), their structures 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 upon severe heat treatment in protein molecules (Feeney, 1975). Thus, covalent bonds such as those mentioned above may be formed among EWP components by dry-heating in the absence and presence of MP and pyrophosphate.

Functional properties of phosphorylated EWP

To examine the stability of EWP against heat-induced insolubility at pH 7.0, 1 g/L solutions of EWP samples dissolved in a 50 mM Tris-HCl buffer (pH 7.0) were heated at various temperatures (60–95°C) for 10 min, and soluble proteins were determined. As shown in Figure 2, soluble protein in the N-, DH-, and MP-EWP solutions decreased markedly as heating temperature increased to >70°C, and then declined to approximately ~18.0% for N-EWP, ~17.6% for DH-EWP, and ~18.5% for MP-EWP by heating at 80°C. The soluble protein after heating at 80°C was 92.0% for PP-EWP, which was higher than those in N-, DH-, and MP-EWP, but most of the proteins were insolubilized at temperature of 95°C. However, under the same heat treatment temperature (95°C), the soluble protein in PP-MP-EWP solution was 76.1%. These results suggested that the stability of EWP against heating at pH 7.0 was considerably improved by phosphorylation alone, and further improved by phosphorylation after glycation. The improved heat stability of food proteins through the

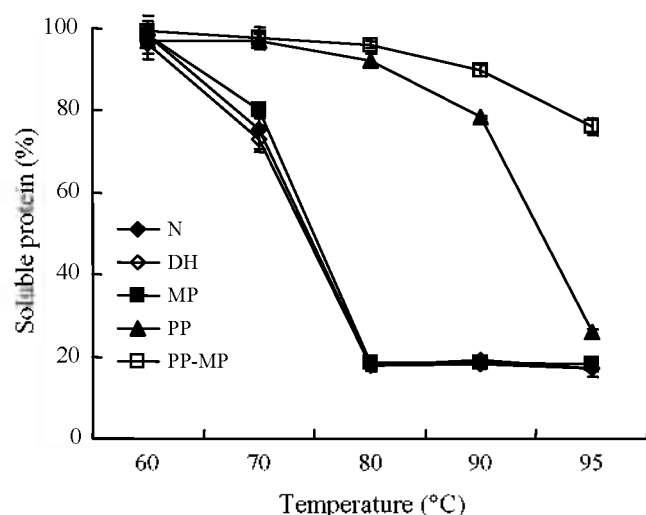


Figure 2. Stability against heat-induced insolubility of EWPs at various temperatures. Protein sample was 1 g/L in 50 mM Tris-HCl buffer (pH 7.0) and heated at 60-95°C for 10 min. Each value is mean \pm SD (n = 3). For N, DH, MP, PP, PP-MP; see Figure 1.

Maillard reaction has been reported by some investigators (Kato et al., 1989; Aoki et al., 1999; Matsudomi et al., 2002). Kato et al. (1995) reported that the heat stability of OVA was more improved by conjugation with glucose-6-phosphate than by conjugation with glucose, suggesting that phosphate groups played an important role in improving the stability of OVA against heat. It has been reported that the electrostatic-repulsive force is important in helping to prevent the random aggregation of denatured food proteins (Kitabatake et al., 1988; Li et al., 2005b; Hayashi et al., 2008).

The antigenicity of OVA and OVM in EWP samples was evaluated by measuring the reactivity of anti-OVA and OVM monoclonal antibody (IgG1) with the antigen (OVA and OVM) adsorbed to the solid phase of a microtitration plate by noncompetitive ELISA. As shown in Figure 3, the reactivity of the OVA and OVM in EWP samples was hardly affected by dry-heating in the absence of MP and pyrophosphate. However, the reactivity of the OVA was reduced significantly by glycation alone and phosphorylation alone, and further reduced by phosphorylation after glycation (Figure 3a), and the reactivity of OVM was reduced significantly by glycation alone, phosphorylation alone and phosphorylation after glycation (Figure 3b). These reductions in antigenicity of OVA and OVM in the PP-MP-EWP were considered to be due to shielding of the epitope by conjugation with MP and unfolding of the epitope by the electrostatic-repulsive force of the introduced phosphate groups (Enomoto et al., 2007, 2008).

The solubilization of the calcium phosphate of EWP was examined using the method for artificial casein

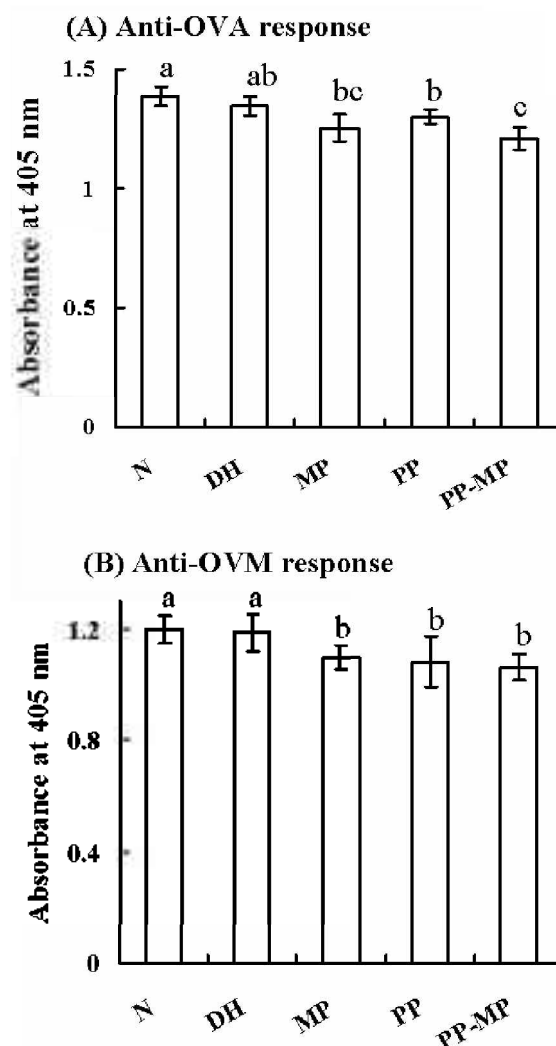


Figure 3. Antigenicity of OVA and OVM in EWPs. (A) The anti-OVA response, and (B) anti-OVM response was evaluated by noncompetitive ELISA, and the results are shown as ELISA values (absorbance at 405 nm). Each value is the mean \pm SD (n = 5). Values with different letters are significantly different ($p < 0.05$) as determined by Student's *t*-test. N, DH, MP, PP, PP-MP; see Figure 1.

micelles, where the final concentrations of calcium, Pi, and citrate were 30, 22, and 10 mM, respectively. As shown in Figure 4, although N-, DH-, and MP-EWP had only a slight calcium phosphate-solubilizing ability, it was enhanced by phosphorylation alone and phosphorylation after glycation. In the presence of 2% protein, PP-EWP solubilized 14.2 mM Pi and 20.4 mM Ca, and PP-MP-EWP solubilized 14.5 mM Pi and 20.7 mM Ca, showing that the calcium phosphate-solubilizing ability of EWP was efficiently enhanced by both phosphorylation methods. Thus, PP- and PP-MP-EWP may be expected to enhance the absorption of calcium.

In conclusion, we have shown that glycation with MP through the Maillard reaction and subsequent

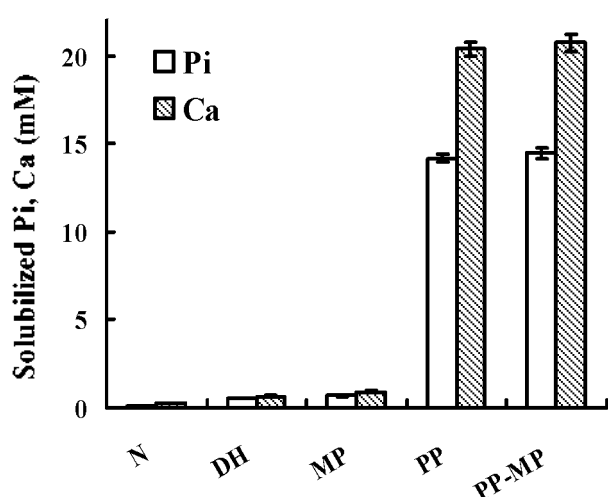


Figure 4. Calcium phosphate-solubilizing ability of EWPs. Test solution contained 20 g/L protein, 30 mM calcium, 22 mM Pi, and 10 mM citrate, with pH adjusted to 6.7 with 1 N KOH. Each column shows the mean \pm SD (n = 3). For definitions of N, DH, MP, PP, and PP-MP: see Figure 1.

phosphorylation by dry-heating in the presence of pyrophosphate was more effective than phosphorylation alone to phosphorylate the EWP. The heat stability of EWP was improved by phosphorylation alone and further by phosphorylation after glycation. The antigenicity of OVA and OVM in EWP was reduced significantly by glycation alone, phosphorylation alone and phosphorylation after glycation. The calcium phosphate solubilizing ability of EWP was enhanced by both phosphorylation methods.

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