



Improvement of Functional Properties of Ovotransferrin by Phosphorylation through Dry-heating in the Presence of Pyrophosphate*

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ABSTRACT : Ovotransferrin (OTf) was phosphorylated by dry-heating in the presence of pyrophosphate at pH 4.0 and 85°C for 1 and 5 d, and the functional properties of phosphorylated OTf (PP-OTf) were investigated. The phosphorus content of OTf increased to 0.91% as a result of phosphorylation and the electrophoretic mobility of PP-OTf also increased. Although the solubility of dry-heated OTf slightly decreased, the decrease was reduced by phosphorylation. The stability against heat-induced insolubilization of OTf was somewhat improved by phosphorylation, but more than 70% of PP-OTf was insolubilized when it was heated at 70°C for 10 min at pH 7.0. However, heat-induced insolubilization of PP-OTf was reduced when it was heated in the presence of phosphorylated ovalbumin. This may explain the excellent stability of phosphorylated egg white protein against heat-induced insolubilization which was reported previously. The emulsifying property of OTf was also somewhat improved by phosphorylation. The calcium phosphate-solubilizing ability of PP-OTf was enhanced. Although the degree of phosphorylation of OTf by dry-heating in the presence of pyrophosphate was similar to that of ovalbumin, the improvement of properties of PP-OTf was considerably different from those of phosphorylated ovalbumin. (**Key Words :** Ovotransferrin, Phosphorylation, Dry-heating, Functional Property)

INTRODUCTION

One useful method of improving the functional properties of food proteins is phosphorylation. The solubility, emulsifying properties, and heat stability of food proteins have been improved by phosphorylation. Several chemical and enzymatic phosphorylation methods have been developed (Matheis and Whitaker, 1984; Seguro and Motoki, 1989; Matheis, 1991; Campbell et al., 1992; Sitohy et al., 1995). Phosphorylation by conjugation of glucose-6-

phosphate through the Maillard reaction was also reported (Aoki et al., 1994, 1997; Kato et al., 1995).

However, there are some problems with these phosphorylation methods (Li et al., 2003). We have phosphorylated egg white protein (EWP) by dry-heating in the presence of phosphate, and the functional properties, such as heat stability and emulsifying properties, of phosphorylated EWP (PP-EWP) were markedly improved (Li et al., 2003, 2004). To further understand the functional properties of PP-EWP, it is important to elucidate its constituents. Ovalbumin (OVA) is the main constituent of EWP, and it is predominantly its behavior, which affects the functional properties of EWP. In a previous paper (Li et al., 2005), we investigated the structural and functional properties of OVA (PP-OVA) phosphorylated by dry-heating in the presence of pyrophosphate.

Ovotransferrin (OTf) is the second major constituent (12%) of EWP and its molecular weight is 78,000 with 683 amino acids. OTf is the most heat-labile constituent in EWP, and heating egg white even at a lower temperature near 60°C caused the coagulation of OTf (Matsuda et al., 1981; Yamashita et al., 1998). In the present study, we phosphorylated OTf (PP-OTf) by dry-heating in the

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presence of pyrophosphate and examined some functional properties of PP-OTf.

MATERIALS AND METHODS

Materials

OTf was purchased from SIGMA-Aldrich (Tokyo, Japan). OVA was purified according to the method of Li et al. (2004) as described below. Egg white was separated from infertile eggs purchased from Manji 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. OVA was purified 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, UK) 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. All reagents used were of analytical grade.

Preparation of phosphorylated proteins

PP-OTf was prepared according to the method given in a previous paper (Li et al., 2005). OTf 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 d. Dry-heated samples were dissolved and dialyzed to remove free pyrophosphate for 3 d against Milli-Q water and then lyophilized. In comparison with PP-OTf, dry-heated OTf (DH-OTf) was prepared as follows: OTf was dissolved at a concentration of 2% in Milli-Q 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-OTf. Finally, dry-heated samples were dissolved and dialyzed for 3 d against Milli-Q water, and then lyophilized.

PP-OVA was prepared by the same method as for PP-OTf.

Determination of phosphorus content (P content) of PP-OTf

Protein samples were digested in perchloric acid. Phosphorus in the digest was regarded as the total phosphorus of PP-OTf. 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 by 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.

Electrophoresis

Native polyacrylamide gel electrophoresis (native-PAGE) was performed using 10% polyacrylamide gels in the absence of sodium dodecyl sulfate (SDS), and SDS-PAGE was performed using 12.5% polyacrylamide gels containing 1.7% SDS under both reducing and nonreducing conditions in the presence and absence of 2-mercaptoethanol (2-ME) according to the method of Leammli (1970). The gels were stained in Coomassie Brilliant Blue R-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 a pump L-2130 chromatograph (Hitachi Ltd, Tokyo, Japan), using a TSKgel BioAssist G4SW_{XL} column (7.8 mm×30 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 50 µl of sample solution (1 mg of protein/ml) filtrated by a 0.45 µm filter syringe 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.

Measurement of solubility

Protein samples were dissolved at a concentration of 1 g/L in 50 mM Tris-HCl buffer (pH 7.0). The solutions (1 ml) were added to 3 ml of the alkali solution (pH 11.0), and defined as total protein solutions. The solutions (1 ml) were then centrifuged at 1,000 g for 20 min, added to 3 ml of the alkali solution (pH 11.0), and defined as soluble protein solutions. Solubility (%) was estimated as the ratio of soluble protein solutions to total protein solutions measured at 280 nm.

Measurements of the stability of protein samples 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 small test tubes stoppered with glass beads and were heated in a water bath at 50 to 90°C for 10 min. Aggregates were precipitated by centrifuging at 1,000 g for 20 min. The soluble protein in the supernatant was measured according to the method of Lowry et al. (1951) to estimate the protein concentration of the solution. The heat stability described in this section means the solubility of OTf after heat treatment.

Measurement of emulsifying properties

The emulsifying properties of protein samples were measured by the method of Pearce and Kinsella (1978). 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

Table 1. Phosphorus content and solubility of N-, DH-, and PP-OTf

Protein ¹	P content ² (%)	Solubility ² (%)
N-OTf	0.00±0.00	100.0±0.0
DH-OTf-1d	0.00±0.00	96.6±1.0
DH-OTf-5d	0.00±0.00	90.9±0.3
PP-OTf-1d	0.66±0.02	97.6±0.3
PP-OTf-5d	0.91±0.01	96.9±0.6

¹ N-OTf, native OTf; DH-OTf-1d and -5d, OTf prepared by dry-heating at pH 4.0 and 85°C for 1 and 5 d in the absence of pyrophosphate; PP-OTf-1d and -5d, OTf prepared by dry-heating at pH 4.0 and 85°C for 1 and 5 d in the presence of pyrophosphate.

² Each value is the mean with its SE (n = 3).

homogenized at 12,000 rpm at 20°C for 1 min with a PT10-35 Polytron homogenizer (Kinematic Ag. Lucerne, Switzerland). From the bottom of each 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 determining the half time of the turbidity measured immediately after emulsion formation.

Measurement of solubilization of calcium phosphate

The preparation of test solutions was conducted according to the procedures for artificial casein micelles (Aoki, 1989). Five hundred microliters of 0.2 M potassium citrate, 500 µl of 0.2 M CaCl₂, and 600 µl of 0.2 M K₂HPO₄ were added to 5 ml of 4% protein solution, followed by the addition of 500 µl of 0.2 M CaCl₂ and 250 µl of 0.2 M K₂HPO₄. The addition of 500 µl of 0.2 M CaCl₂ and 250 µl of 0.2 M K₂HPO₄ was repeated to yield calcium and Pi concentrations 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 10 ml by measuring the weight of 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 Pi in the supernatant were then determined. Calcium was determined with a Hitachi Z-600 atomic absorption spectrophotometer (Hitachi Ltd, Tokyo, Japan).

Representation of data

The data were expressed as mean values of three replicated determinations with standard error (SE).

RESULTS AND DISCUSSION

Characteristics of PP-OTf

OTf was phosphorylated by dry-heating at pH 4.0 and 85°C for 1 and 5 d in the presence of pyrophosphate. As

shown in Table 1, the P content of PP-OTf was 0.66% by dry-heating at pH 4.0 and 85°C for 1 d (PP-OTf-1d), and increased to 0.91% by dry-heating for 5 d (PP-OTf-5 d). Phosphorus was not detected in OTf. Therefore these results suggest that phosphorylation occurred efficiently in OTf, as well as phosphorylation of EWP and OVA (Li et al., 2004, 2005).

The solubility of food protein is an important property for its application in food processing. The effect of the phosphorylation on the solubility of OTf was measured at pH 7.0. Although the solubility of OTf decreased somewhat as a result of dry-heating in the absence of pyrophosphate for 5 d, it was improved by phosphorylation.

Figure 1A shows the native PAGE pattern of native (N-), DH-, and PP-OTf. In the absence of pyrophosphate, there were almost no changes in the mobility of OTf. However, the mobility of OTf increased by dry-heating in the presence of pyrophosphate, and the changes in the mobility corresponded to the phosphorylation level. The phosphorylation level of PP-OTf is close to that of PP-EWP and PP-OVA, as reported in the previous paper (Li et al., 2004, 2005), by dry-heating under the same conditions. The mobility of protein increased with an increase in dry-heating time from 1 to 5 d in the presence of pyrophosphate. These results indicate that a higher level of negatively charged phosphate groups on OTf caused greater mobility.

From the native PAGE pattern of OTf, aggregation of OTf as a result of dry-heating was observed. To assess the binding type of aggregates, SDS-PAGE in the absence and presence of 2-ME, respectively, was performed (Figure 1B). It can be clearly seen from Figure 1B that the dry-heating induced aggregation in the protein, showing polymeric proteins. Disulfide bonds of OTfs were cleaved in the presence of 2-ME, but there was only slight dissociation. The results indicate that covalent bonds other than disulfide were formed by dry-heating. These bonds formed in proteins by dry-heating have been discussed by some researchers (Kato et al., 1989; Watanabe et al., 1999), but 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 in OTf on dry-heating in the absence and presence of pyrophosphate. On the other hand, it was also observed that the mobility of PP-OTf was slightly slower than that of DH-OTf in the absence and presence of 2-ME, suggesting a slight increase of the molecular mass of OTf by phosphorylation.

To examine the change of molecular character in PP-OTf, gel permeation mode HPLC was performed (Figure 2).

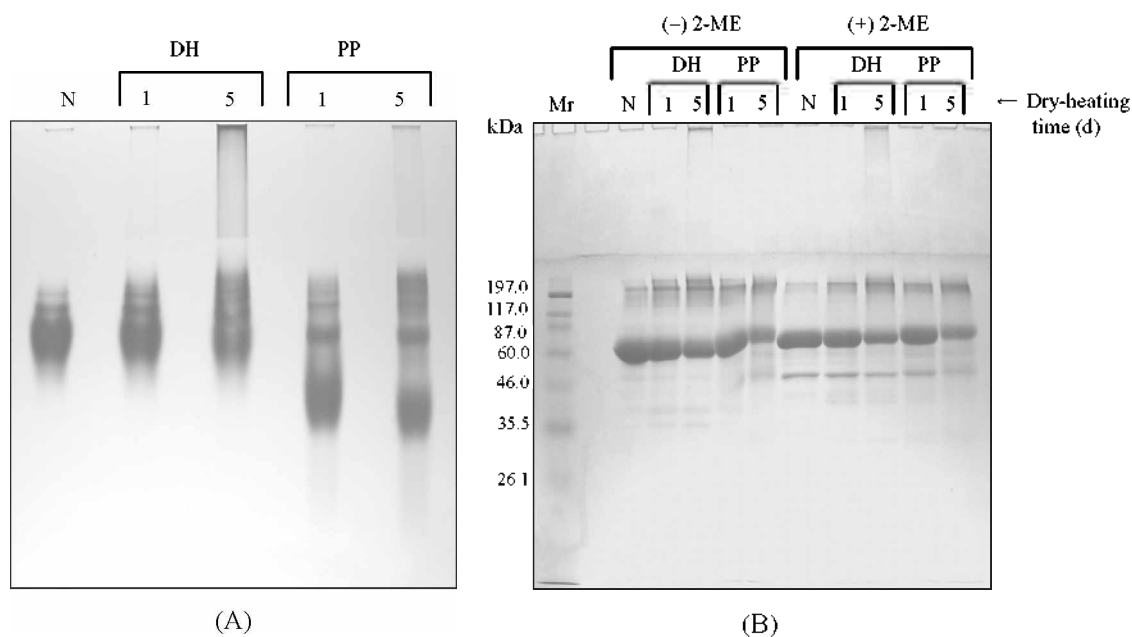


Figure 1. Electrophoretic patterns of OTfs: (A) Native PAGE (10% polyacrylamide gel in the absence of SDS), (B) SDS-PAGE (12.5% polyacrylamide gel in the presence of SDS) with (+) and without (-) 2-mercaptoethanol (2-ME). Mr, marker protein; N-OTf, native OTf; DH-OTf-1d and -5d, OTf prepared by dry-heating at pH 4.0 and 85°C for 1 and 5 d in the absence of pyrophosphate; PP-OTf-1d and -5d, OTf prepared by dry-heating at pH 4.0 and 85°C for 1 and 5 d in the presence of pyrophosphate.

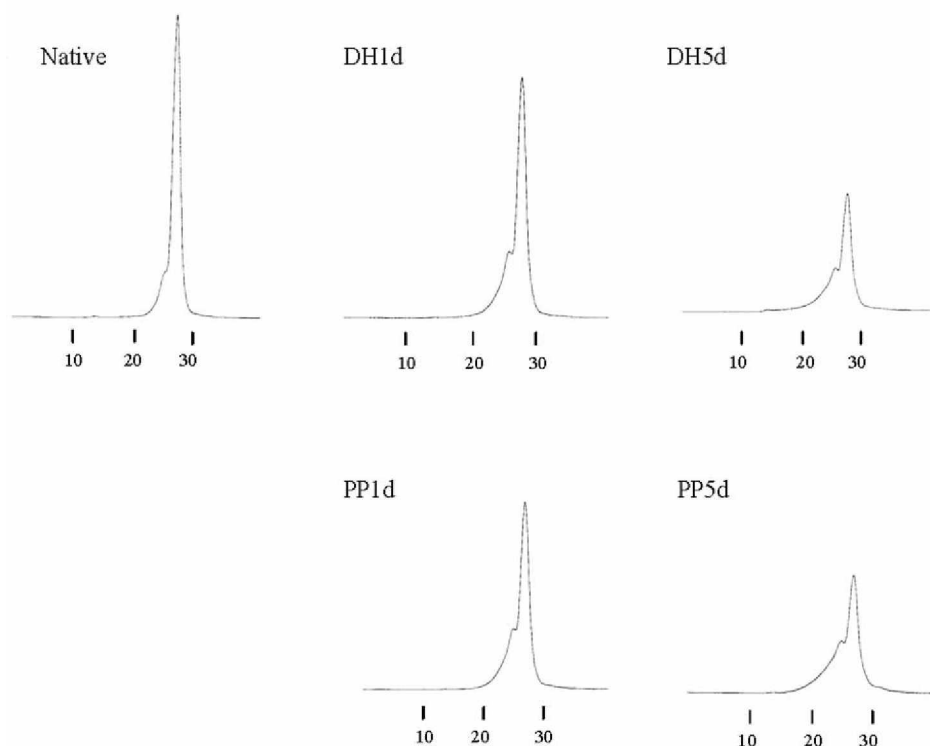


Figure 2. HPLC patterns of OTfs from a TSKgel BioAssist G4SW_{XL} column (7.8 mm×30 cm). Elution buffer was 0.1 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl; flow rate = 0.5 ml/min. N-, DH-, and PP-OTf: see Figure 1.

Dimeric form was observed in DH- and PP-OTf. The peak of OTf monomer became broader by dry-heating especially in the presence of pyrophosphate (PP-OTf-5d), suggesting that dry-heating and phosphorylation caused the changes in the molecular form of OTf.

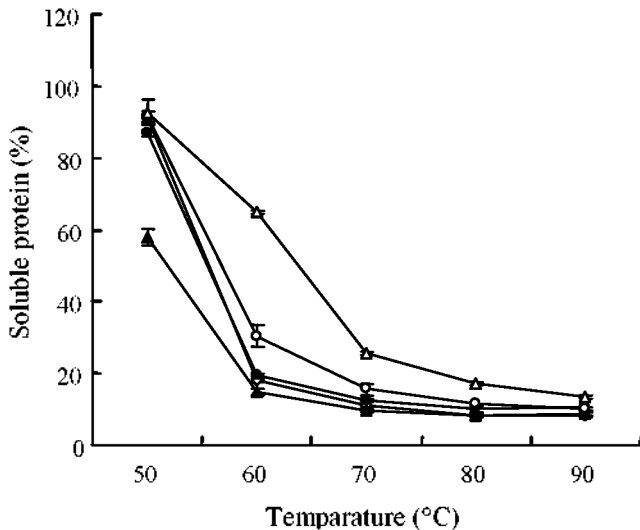


Figure 3. Stability against heat-induced insolubility of OTfs at various temperatures. The protein sample was 1 g/L in 50 mM Tris-HCl buffer (pH 7.0) and heated at 50 to 90°C for 10 min. (◇) N-OTf, (●) DH-OTf-1d, (▲) DH-OTf-5d, (○) PP-OTf-1d and (△) PP-OTf-5d; see Figure 1. Each value is the mean with its SE (n = 3).

Functional properties of phosphorylated OTf

To examine the stability of OTf against heat-induced insolubility at pH 7.0, 0.1% solutions of N-OTf, DH-OTf and PP-OTf dissolved in a 50 mM Tris-HCl buffer (pH 7.0) were heated at various temperatures (50 to 90°C) for 10 min, and the soluble proteins were determined. OTf is the most instable protein against heat treatment in EWP, and heating of egg white even at a lower temperature near 60°C caused aggregation of OTf (Matsuda et al., 1981; Yamashita et al., 1998). As shown in Figure 3, the soluble proteins of N-OTf and DH-OTf (1 and 5 d) decreased markedly as heating temperatures increased over 60°C and decreased by around 10.5% at 80°C. In the case of PP-OTf-5d, the soluble protein was 65% when heated at 60°C, whereas the soluble proteins decreased markedly when heated to temperatures in excess of 70°C.

The results suggest that stability of OTf against heating at pH 7.0 was somewhat improved by phosphorylation, suggesting that introduced phosphate groups played a role in improving the heat stability of OTf. In the previous paper (Li et al., 2004), we reported that most PP-EWP remained soluble when its solution was heated at pH 7.0 and 90°C for 10 min. This suggests that PP-OVA may stabilize the PP-OTf like a chaperon when PP-EWP solution is heated. To confirm this, the heat stability of the mixed solution of PP-OTf and PP-OVA was examined. The mixtures of PP-OTf and PP-OVA in the ratio of 10:90, 20:80, and 50:50 were heated at 60 to 80°C for 10 min, and the soluble proteins measured. The results are shown in Figure 4; dotted lines in the columns are estimated values when PP-OTf and PP-

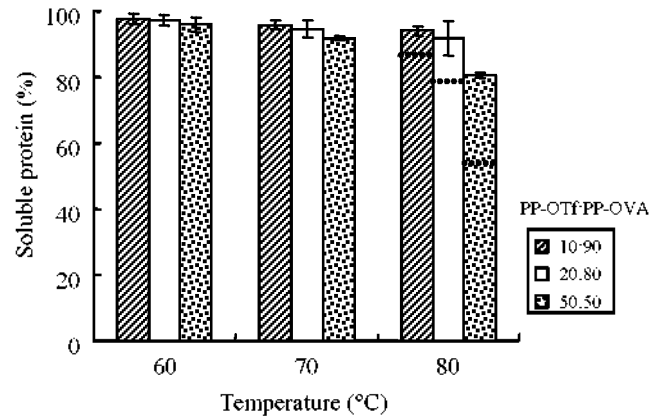


Figure 4. Stability of mixed PP-OTf-5d and PP-OVA-5d against heat-induced insolubility. PP-OTf and PP-OVA were mixed in the proportion of 10:90, 20:80, and 50:50, and then heated at 60 to 80°C for 10 min. N-, DH-, and PP-OTf: see Figure 1. Dotted lines on the columns were estimated values when PP-OTf and PP-OVA were heated independently. Each column shows the mean with its SE (n = 3).

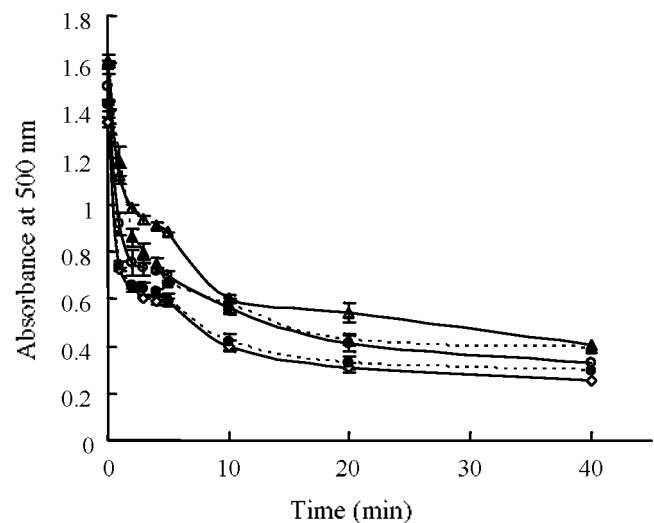


Figure 5. Emulsifying properties given as turbidity (A 500 nm) of OTfs as a function of standing time after emulsification. The turbidity of the emulsion is plotted as the ordinate and standing time after emulsion formation as the abscissa. (◇) N-OTf, (●) DH-OTf-1d, (▲) DH-OTf-5d, (○) PP-OTf-1d and (△) PP-OTf-5d; see Figure 1. Each value is the mean with its SE (n = 3).

OVA were heated independently. When the mixture of 50:50 was heated at 80°C, the soluble protein accounted for 80.5%, which was much higher than the estimated value when both proteins were heated independently. This may explain the excellent heat stability of PP-EWP even though it contains the less heat-stable PP-OTf. As described above, the degree of improvement of heat stability of OTf by phosphorylation was much smaller than that of OVA.

Figure 5 shows the emulsifying properties of N-, DH-, and PP-OTf. The value of the ordinate at zero time denotes

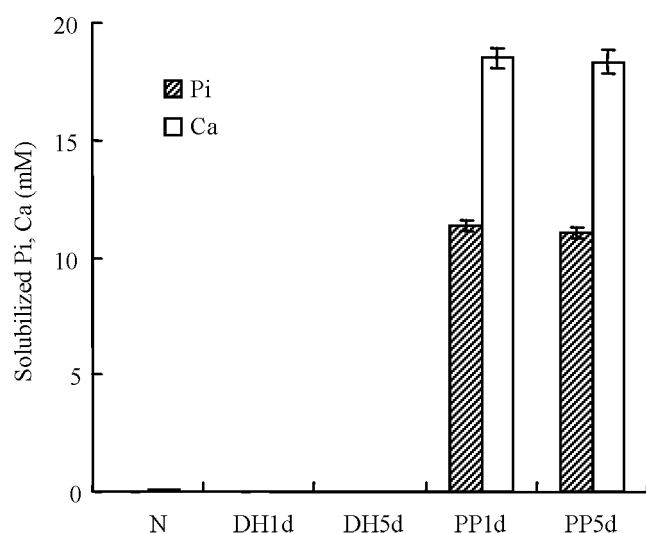


Figure 6. Calcium phosphate-solubilizing ability of OTfs. The test solution contained 2% protein, 30 mM Ca, 22 mM Pi, and 10 mM citrate, and its pH was adjusted to 6.7 with 1 N KOH. N-, DH-, and PP-OTf: see Figure 1. Each column shows the mean with its SE (n = 3).

the relative emulsifying activity, and the half-life of initial turbidity reflects the stability of the emulsion. There were only small differences in relative emulsifying activity and stability between N- and DH-OTf. However, the emulsifying activity of PP-OTf-5d at 500 nm was 1.61, which was 1.2 times that of N-OTf. In addition, the turbidity of emulsion from PP-OTf-5d was a little higher than that from N- and DH-OTf-5d. Therefore, the emulsifying stability of PP-OTf-5d was 6.3 min, which was 4.2 and 5.3 times longer than N- and DH-OTf-5d, respectively. This suggests that the emulsifying property of OTf was somewhat improved by phosphorylation.

The solubilization of calcium phosphate by OTf was examined using the method of artificial casein micelles, where the final concentrations of calcium, Pi, and citrate were 30, 22, and 10 mM, respectively. The solubilized calcium and Pi were estimated from the difference between their soluble concentrations in the solutions with and without protein. As shown in Figure 6, although N-, DH-OTf had only a slight calcium phosphate-solubilizing ability, it was enhanced by phosphorylation. In the presence of 2% protein, PP-OTf-5d solubilized 18.3 mM calcium and 11.0 mM Pi, showing that the calcium phosphate-solubilizing ability of OTf was efficiently enhanced by phosphorylation. Thus, PP-OTf may be expected to enhance the absorption of calcium.

In summary, in the present study, as with OVA, OTf was effectively phosphorylated by dry-heating in the presence of pyrophosphate, but the effect of phosphorylation of OTf on the improvement of heat stability and emulsifying property was smaller than that on OVA. On the other hand, the

calcium phosphate-solubilizing ability of OTf was effectively enhanced by phosphorylation.

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