Modification of Ovalbumin with Glucose 6-Phosphate by Amino–Carbonyl Reaction. Improvement of Protein Heat Stability and Emulsifying Activity

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Ovalbumin (OVA) was modified with glucose (Glu) and glucose 6-phosphate (G6P) through the amino–carbonyl reaction (Maillard reaction), and heat-induced aggregation and emulsifying activity of the modified proteins were investigated. G6P reacted with the free amino groups in a similar manner to Glu; about 70–80% of the total amino groups were blocked by the reaction at 50 °C, and 65% relative humidity for 3 days. However, the reaction with G6P induced protein polymerization and brown-color development more strongly than that of Glu. OVA modified with G6P was much more acidic than either native OVA or the Glu-modified OVA, and it was highly soluble and quite resistant to heat-induced aggregation, i.e., its high-concentration solution (5%) was still completely soluble and transparent even after being heated at 100 °C for 10 min. Furthermore, OVA emulsifying activity was increased about 5-fold by the modification with G6P.

Keywords: *Glucose 6-phosphate; ovalbumin; protein glycation; protein modification; amino–carbonyl reaction; Maillard reaction; emulsifying activity; heat stability*

INTRODUCTION

Protein phosphorylation has also been used for changing the functional properties of food proteins. Of the phosphorylation reagents tested so far, only $POCl_3$ and sodium trimetaphosphate (STMP) might be used as economical and practical reagents for large-scale application to food. Water solubility of soybean protein phosphorylated with STMP was increased, though phosphorylation with STMP required alkaline pH. Proteins undergo various physical and chemical changes in alkaline solutions, including lysinoalanine formation and racemization of certain amino acids. On the other hand, phosphorylation of proteins with $POCl_3$ can be done at mild conditions, though protein cross-linking also occurs, resulting in the decrease in water solubility of the modified proteins (Matheis and Whitaker, 1984).

We have reported that an amino–carbonyl reaction (Maillard reaction) with reducing sugars under mild conditions is useful for the protein modification to improve some protein functionalities such as water solubility and heat stability. OVA–Glu complexes formed in the early stage of the Maillard reaction were easily solubulized and possessed a marked resistance to conformational destruction and heat coagulation (Kato et al., 1981, 1983). We have also reported on the amino–carbonyl reaction between protein and various reducing sugars with structural analogy and have found that structural and chemical properties of sugars affected the proceeding of Maillard reaction and the properties of the reaction products of protein–sugar complexes (Kato et al., 1986, 1988, 1989). G6P is a main metabolic product of sugars and has a hemiacetal bond of carbonyl group. Hence, G6P with both carbonyl and phosphoryl groups might be used as a safe protein modifier to introduce phosphoryl groups into food proteins by the amino–carbonyl reaction as a mild and safe modification method.

Aims of the present study are to elucidate how G6P reacts with protein lysine residues and how functional the properties of the modified proteins are. In this paper, some characteristic properties of G6P-modified OVA are described on additional negative charges, intermolecular cross-linking, heat stability, and emulsifying activity. In addition, these properties of G6P-modified OVA are compared with those of unmodified and Glu-modified OVAs.

MATERIALS AND METHODS

Modification of OVA with Glu or G6P. OVA was prepared from fresh egg white of White Leghorn hens by the ammonium sulfate precipitation method (Marshall and Neuberger, 1972). Glu and G6P were obtained from Wako Pure Chemical Ltd. (Osaka).

OVA and Glu (1:0.5 w/w) and OVA and G6P (1:0.94 w/w) were dissolved in distilled water at protein concentration of 2 mg/mL, and the pH of the solution was adjusted to 7.0 with diluted NaOH solution. Samples of the mixed solution were pipetted into each tube (1 mg of protein/tube), and the separated samples were then freeze-dried. The dried samples were kept in desiccators at 50 °C and 65% relative humidity (RH) for various periods (0–5 days) to accelerate the amino–carbonyl reaction (Kato et al., 1989) and then kept at −20 °C until use.

Electrophoresis. Polyacrylamide gel electrophoresis (7.5% acrylamide) and sodium dodecyl sulfate (NaDodSO$_4$) polyacrylamide gel electrophoresis (8% acrylamide) (SDS–PAGE) were performed according to the

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methods of Davis (1964) and Laemmli (1970), respectively, with subsequent Coomassie Blue staining.

Measurement of Browning and Free Amino Group Content. Browning of protein–G6P or protein–Glu mixture by the amino–carbonyl reaction was measured by absorbance at 420 nm of the water solution (1 mg of protein/mL).

Free amino groups were measured by the fluorometric method using fluorescamin (Sigma) according to Böhlen et al. (1973). The fluorescence was determined with the excitation at 390 nm and emission at 485 nm. The free amino group was expressed as percentage of fluorescence intensity per milligram protein against native OVA.

High-Performance Gel Chromatography. High-performance gel chromatography was carried out at room temperature (22–25 °C) with Shimadzu LC-5A chromatograph equipped with a Shimadzu SPD-2A spectrophotometric detector, using a TSK-GEL G3000sw column. Samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl, and 25 mL of the sample solution (1 mg of protein/mL) was injected. The samples were eluted with the same buffer solution at flow rate of 0.5 mL/min, and the elution profile was monitored by UV absorbance at 280 nm. The peak area of the elution profiles was determined by a HITACHI integrator Model D-2500.

Heat Stability. OVA, OVA–Glu, and OVA–G6P were dissolved at a protein concentration of 10 mg/mL of 50 mM Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer to remove free Glu or G6P. These samples were diluted to 1 mg of protein/mL with the same buffer and heated at 60–100 °C for 10 min. Aggregates were precipitated by centrifugation at 1500*g* for 20 min. The UV absorbance at 280 nm of the supernatant was measured to estimate protein concentration of the solution.

OVA–Glu and OVA–G6P were treated for 1 day, and the mixture of OVA with Glu or G6P (1:0.5 or 1:0.94 w/w) was dissolved at various protein concentrations of 0.05, 0.1, 0.5, 1.0, 3.0, and 5.0% in distilled water, and the pH of each sample solution was adjusted to 7.0 with diluted NaOH solution. These samples were heated in boiling water for 10 min, and aggregation was observed by visual inspection.

Emulsifying Property. One hundred and fifty microliters of peanut oil was added to 100 μL of OVA–Glu or OVA–G6P solution (1 mg of protein/mL), and each sample was mixed well and then emulsified by being mixed with a homogenizer (Vortex Genie) at the highest revolution for 1 min. Immediately after the mixtures were emulsified, aliquots of the emulsion were diluted 30 times with 0.1% $NaDodSO_4$, and the absorbance of the diluted emulsion was then determined in a 1-cm path length cuvette at a wavelength of 500 nm according to the method of Pearce and Kinsella (1978).

RESULTS AND DISCUSSION

Characteristics of the Amino–Carbonyl Reaction of OVA with G6P. The decrease in free amino group was measured for OVAs modified with G6P or Glu and without sugars for 5 days at 50 °C and 65% RH. The changes in relative value of free amino group content are shown in Figure 1. The free amino group decreased quickly in samples treated with sugars, and about 50% of the free amino group was blocked with G6P or Glu after 1 day of treatment, whereas no decrease was observed for OVA treated without sugars.

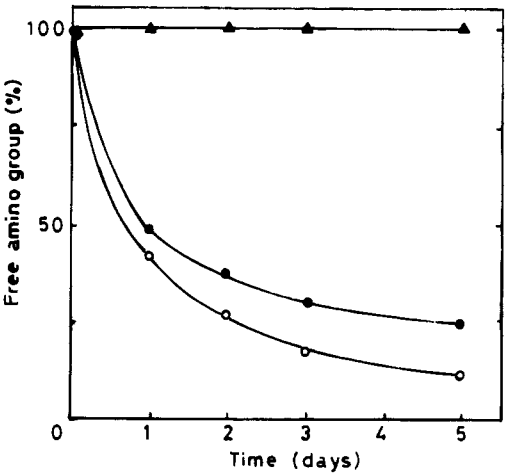

Figure 1. Decrease in free amino groups of OVA with G6P and Glu. OVA was treated with G6P (●) or Glu (○) at 50 °C and 65% RH for 1–5 days. OVA (▲) was treated in the absence of sugars at the same condition for comparison. Free amino group represents relative fluorescence intensity (%) against native OVA.

OVA has 20 lysine residues per molecule. Therefore, the OVA–G6P treated for 1 day is expected to already have no less than 10 phosphoryl groups through lysine-ε-amino groups, assuming that no degradation of reacted G6P molecules occurred. The rate of decrease in OVA amino groups by the reaction with G6P was a little lower than that of decrease by the reaction with Glu.

Modification efficiency of OVA amino groups by a chemical phosphorylation with phosphorus oxchloride ($POCl_3$) was reported to be about 40% (Heidelberg et al., 1941). Casein and lysozyme were also reported to be phoshorylated by phosphorus oxychloride at pH 6–8 and 3–20 °C, and up to 7.4 and 6.2 mol of phosphoryl groups were covalently attached to one of casein and lysozyme molecules, respectively (Matheis et al., 1983). Soybean proteins were enzymatically phosphorylated with the catalytic subunit of cAMP-dependent protein kinase, and the modification efficiency was reported to be about 1.0 mol of phosphate/mol of glycinin (Ross and Bhatnagar, 1989) or 2 mol of phosphate/mol of 11S (Seguro and Motoki, 1990). By protein phosphorylation with G6P through the amino–carbonyl reaction, no less than 10 mol of phosphoryl groups was added to 1 mol of OVA. Hence, for addition of surface negative charges and hydroxyl groups to proteins, the amino–carbonyl reaction with G6P would be a useful protein modification method without serious chemical damage or denaturation of proteins.

Polyacrylamide gel electrophoretic profiles of OVA modified with G6P for 3 or 5 days were compared with those of OVA modified with Glu for the same periods. As shown in Figure 2, the major band of OVA–G6P treated for 3 days already migrated to the gel bottom of the anode side. The electrophoretic mobility of the monomer band of OVA–Glu also increased gradually with increase in treatment periods, but the increase in mobility was smaller than that of OVA–G6P. These results indicate that positive charges of OVA were decreased by amino group blocking with Glu or G6P, whereas negative charges were increased by adding phosphate groups to the modified lysine residues through the reaction with G6P. No changes in electrophoretic profiles were observed for OVA treated without sugars (data not shown).

To estimate intermolecular covalent cross-linking, OVA–Glu and OVA–G6P were analyzed by $NaDodSO_4$

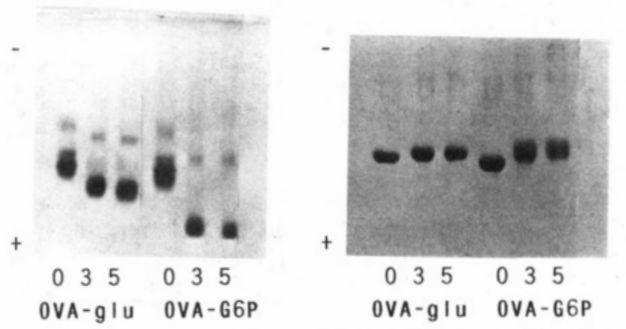

Figure 2. PAGE of OVA modified with Glu or G6P. Left: native PAGE (7.5%acrylamidegel). Right: SDS–PAGE (12.5% acrylamide gel). The samples were analyzed before (0) and 3 or 5 days after the mild heat treatment (50 °C, 65% RH).

gel electrophoresis under a reducing condition. As shown in Figure 2, the stained band corresponding to monomeric OVA–G6P became weaker and broader with an increase in treatment periods, though that of OVA–Glu did not. Protein bands corresponding to high molecular size polymers with low electrophoretic mobility were clearly observed near or at the top of the gel for OVA–G6P treated for 3 days or more.

Protein polymerization during the treatment with Glu and G6P was analyzed by gel filtration HPLC. Figure 3 shows typical elution profiles of both OVA–Glu and OVA–G6P treated for 3 days. Native OVA was eluted at the retention time of about 38 min. Several additional peaks with shorter retention time appeared for samples treated with sugars, and the number and height of the peaks with shorter retention time increased as the treatment time increased, especially in the OVA–G6P system. The peak height corresponding to monomeric and polymeric forms of OVA–G6P was markedly lower and higher than that of OVA–Glu, respectively. The peaks indicated by shaded and unshaded areas were regarded as polymer and monomer fractions, respectively, and relative amount of proteins in each fraction was estimated based on the peak area. The apparent proportions of the polymer fraction for samples treated for one to 5 days are shown in Figure 4. Protein polymers were formed in both OVA–Glu and OVA–G6P systems, but polymers were produced more quickly in OVA–G6P and increased with treatment periods. Polymers produced in OVA–G6P system were 2–5 times more in amount than that of OVA–Glu system.

Figure 4 also shows the brown color formation of OVA–G6P and OVA–Glu by the amino carbonyl reaction. The brown color of OVA–G6P was developed more strongly than that of OVA–Glu, especially at the later stage of treatment period. The absorbance at 420 nm of OVA–G6P treated for 5 days was about 3 times higher than that of OVA–Glu.

Although the rate of free amino group decrease in OVA–G6P system is slower than that of Glu system (Figure 1), the browning reaction and protein polymerization at the later stages of reaction proceeded more rapidly in OVA–G6P than OVA–Glu, suggesting that the polar phosphate group of G6P accelerates, the advanced steps of the amino–carbonyl reaction.

Changes in Functional Properties of OVA by the Modification with G6P. To study the heat stability of OVA modified with G6P, 0.1% solutions of OVA and OVA modified with G6P or Glu (1-day treatment) after heating at various temperatures (60–95 °C) for 10 min, and soluble protein was determined and expressed as its percentage to total protein in the solution. As shown in Figure 5, solubility of OVA without treatment was decreased as heating temperature increased. There was little or no soluble OVA after heating at >85 °C, whereas OVA modified with G6P or Glu for 1 day was still completely soluble after heating at 95 °C for 10 min.

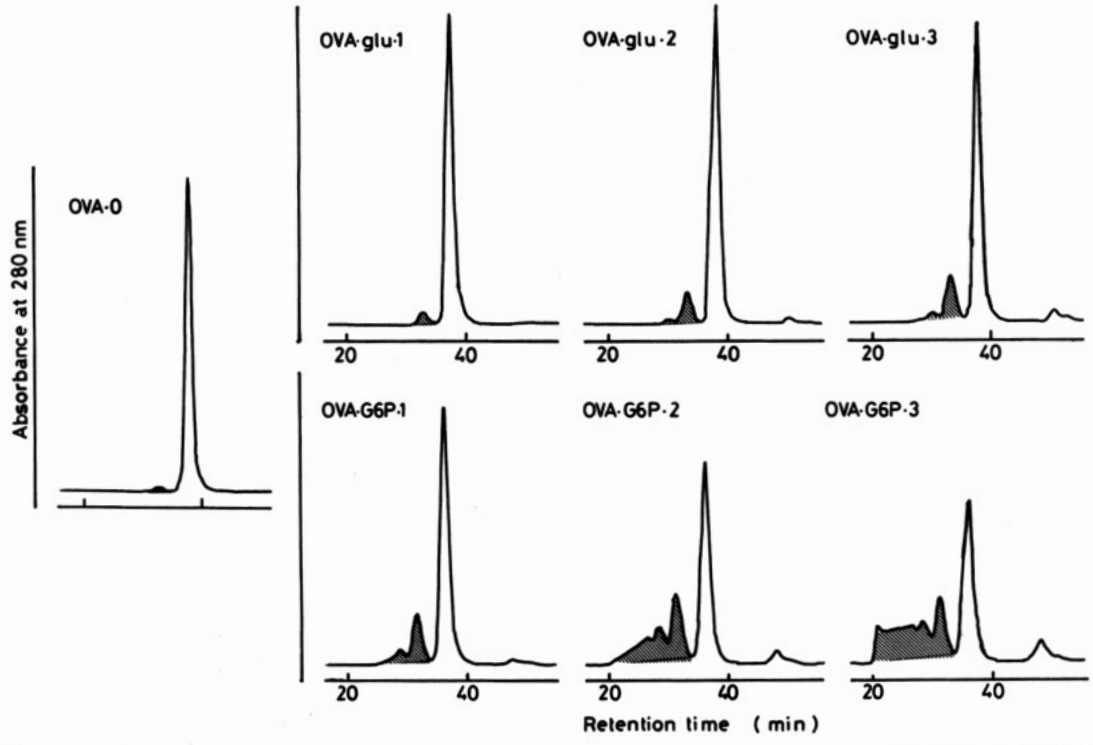

Figure 3. Elution profiles of OVA–G6P, OVA–Glu, and OVA from a TSK-GEL G3000sw column. The flow rate was 0.5 mL/min, and 0.1 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl was used as the effluent. OVA and sugar mixtures were treated at 50 °C and 65% RH for 1–5 days. The typical profiles of samples treated for 1, 2, and 3 days are shown. The elution profile of native OVA (OVA-0) is also shown for comparison.

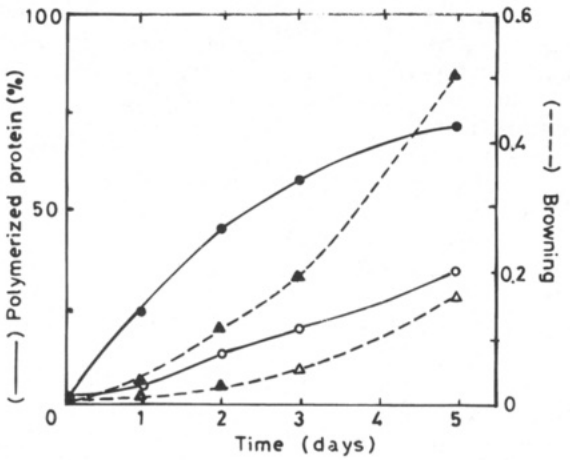

Figure 4. Polymerization and brown-color development of OVA modified with G6P or Glu as estimated from gel filtration profiles and absorbance at 420 nm, respectively. OVA was treated with G6P (●, ▲), or Glu (○, △) for 1–5 days at 50 °C, 65% RH.

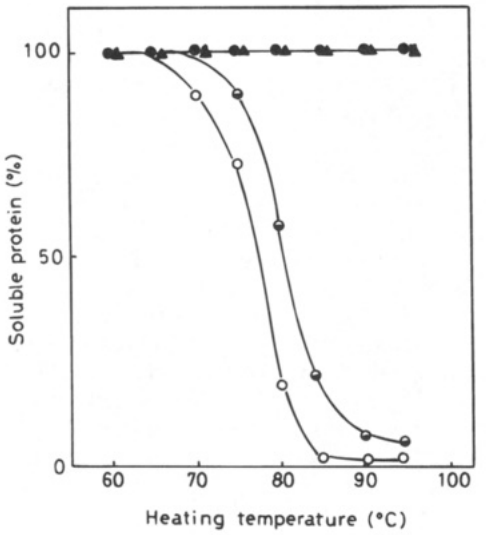

Figure 5. Protective effect of G6P modification on heat-induced aggregation of OVA. OVA–G6P and OVA–Glu were prepared by treatment at 50 °C and 65% RH for 1 day. Free G6P and Glu were removed by dialysis. The samples were dissolved at a concentration of 0.1% in 0.05 M Tris-HCl buffer (pH 7.0). Soluble protein in the solutions of OVA (○), OVAs modified with G6P (●) or Glu (▲) was determined after being heated at various temperatures (60–95 °C) for 10 min and are expressed as relative concentration (%) against unheated ones. The OVA mixed with G6P (1:0.94%) (◒) was also analyzed for comparision.

The presence of free G6P in OVA solution gave only a slight effect on heat stability of OVA.

Food proteins may sometimes be used as solutions of higher concentration, and heat-induced aggregation of protein is largely dependent on protein concentration. Furthermore, removal of unbound G6P by dialysis does not seem to be economical and practical. Therefore, various concentrations (0.05–5.0%) of sample solutions were used without removal Glu or G6P for heat-induced aggregation experiments (heated in boiling water for 10 min) (Figure 6). Protein aggregation was observed for OVA mixed with Glu in all protein concentrations tested (0.05% to 5.0%), whereas OVA treated with Glu for 1 day did not aggregate at the lower concentrations of 0.05 and 0.1%. OVA mixed with G6P (1:0.94 w/w) also did not aggregate under the conditions of lower protein concentrations. Moreover, OVA treated with G6P for

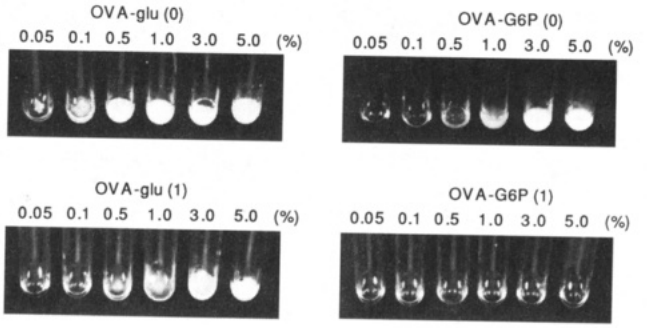

Figure 6. Suppression of heat-induced aggregation of OVA in higher concentration solutions by the amino–carbonyl reaction with G6P. The 1-day-treated protein–sugar complexes, OVA–G6P (1) and OVA–Glu (1), were heated at 100 °C for 10 min at various protein concentration (0.05–5.0% in water solution). The samples without the treatment, OVA–G6P (0) and OVA–Glu (0), were also examined for comparison.

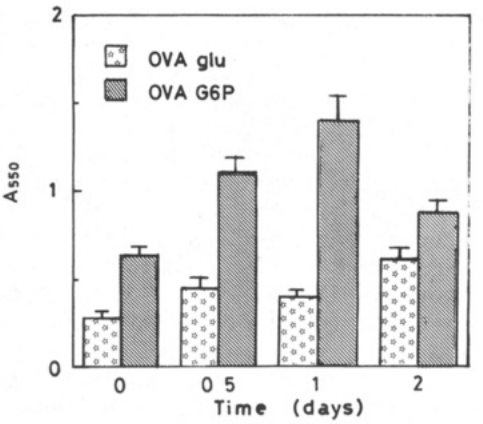

Figure 7. Improvement of emulsifying activity of OVA by the amino–carbonyl reaction with G6P. OVA was mixed with G6P and treated at 50 °C and 65% RH for 2 days, and then the OVA–G6P was used for the emulsifying-activity measurement. OVA modified with glucose (OVA–Glu) was also analyzed for comparison.

1 day was completely soluble at all concentrations tested, and neither protein aggregation nor turbidity was observed even at the highest concentration (5%) tested. Thus, the modification with G6P to OVA strongly suppressed heat-induced aggregation of OVA even at high protein concentration such as 5%.

Functional properties of some phosphorylated proteins have been studied and summarized by Matheis and Whitaker (1984). Woo and Richardson (1983) reported that phosphorylated β-lactoglobulin increased emulsyfying activity, and Heidelberger et al. (1941) showed the loss of heat coagulation. On the other hand, water solubility of some proteins and emulsifying activity of phosphorylated casein with $POCl_3$ were reported to be decreased (Matheis et al., 1983).

Hence, the effect of G6P modification on emulsifying activity of OVA was investigated by using OVAs treated with G6P or Glu for various periods. As shown in Figure 7, the emulsifying activity of OVA–G6P mixture without treatment was a little higher than that of OVA–Glu mixture without treatment, indicating that the presence of free G6P in the protein/oil mixture slightly affected the emulsion formation. The emulsifying activity of native OVA was almost the same as that of the untreated OVA–Glu (data not shown). Emulsifying activity of OVA–G6P increased as the treatment periods increased and reached a maximum at 2 days, whereas that of OVA–Glu was almost constant throughout the treatment period. The activity of OVA–G6P

treated for 1 day was about 2 times higher than that of the untreated one and was about 4 times higher than that of OVA–Glu treated for 1 day.

Improvement of protein functionality by chemical modification might permit wide application of a protein as food ingredients and effective use of unutilized proteins as food proteins. However, acceptability of modification methods to consumer as well as legal regulations of modified proteins as food additives should also be considered. From this viewpoint, protein modification with sugars by the Maillard reaction may have some superiority among various modification methods, since the Maillard reaction naturally occurs in many food and food products currently consumed and sugars and phosphorylated sugars exist widely in plant and animal tissues used as food materials.

In conclusion, results of the present study demonstrated that phosphoryl groups could effectively be added to proteins through a controlled amino carbonyl reaction using G6P as a carbonyl compound, and that the addition of phosphryl groups to proteins, probably as 6-phosphodeoxyfructosyl residues, improved protein functional properties such as solubility, heat stability, and emulsifying activity.

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