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Glycation and Phosphorylation of β-Lactoglobulin by Dry-Heating: Effect on Protein Structure and Some Properties

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β-Lactoglobulin (β-Lg) was glycated with maltopentaose and subsequently phosphorylated by dry-heating in the presence of pyrophosphate to investigate the structural and functional properties of phosphorylated β-Lg. The circular dichroism spectra showed that the change of the secondary structure in the β-Lg molecule by glycation and subsequent phosphorylation was small. The differential scanning calorimetry thermograms of β-Lg showed that the denaturation temperature of the most stable domain was only slightly affected, whereas the retinol-binding activity of β-Lg was somewhat reduced by glycation and subsequent phosphorylation. These results indicated that the conformational changes of the β-Lg molecule by glycation and subsequent phosphorylation were mild. The anti-β-Lg antibody response was somewhat reduced by glycation, but significant changes were not observed by phosphorylation. Although the stability of β-Lg against heat-induced insolubility was improved by glycation alone, it was further enhanced by phosphorylation. The calcium phosphate solubilizing ability of β-Lg was enhanced by phosphorylation following glycation.

KEYWORDS: β-Lactoglobulin; dry-heating; Maillard reaction; phosphorylation; structural properties; functional properties

INTRODUCTION

Phosphorylation has been proven to be a useful method for improving the functional properties of food proteins. The functional properties of some phosphorylated proteins have been studied and reviewed by Matheis and Whitaker (*1*). Over the past few decades, several phosphorylation methods have been reported by some researchers (*2*−*5*). Aoki et al. and Kato et al. (*6*−*8*), for example, reported that β-Lactoglobulin (β-Lg) and ovalbumin were phosphorylated by conjugation of glucose-6-phosphate through the Maillard reaction. However, these phosphorylation methods have posed some problems mentioned in previous papers (*9*, *10*), in which they were considered to be very difficult to put to practical use. In one such paper (*9*), we phosphorylated egg white protein (EWP) and whey protein isolate (WPI) by dry-heating in the presence of phosphate, but WPI showed a lower phosphorylation level than EWP. This was considered to be due to a lower sugar content of WPI. We then attempted to prepare phosphorylated WPI by glycation with maltopentaose (MP) through the Maillard reaction and subsequent phosphorylation by dry-heating in the presence of pyrophosphate (*11*), with the result that some functional properties, such as heat stability, emulsifying properties, and gelling properties, were improved by phosphorylation after glycation. Furthermore, the calcium phosphate solubilizing ability of WPI was enhanced by phosphorylation. Interestingly, a transparent and firmer heat-induced gel of phosphorylated WPI was obtained as reported in a previous paper (*11*).

Although the functional properties, especially the gelling properties, of WPI were improved by dry-heating in the presence of pyrophosphate after glycation, the molecular basis of the structural changes producing the improvement have not yet been sufficiently unraveled. β-Lg is the most abundant whey protein in WPI, and its behavior predominantly affects WPI functional properties. Therefore, monitoring the structural changes of phosphorylated β-Lg (PP−MP-β-Lg) by dry-heating in the presence of pyrophosphate after glycation with MP will be helpful for further understanding of the relationship between the structural and functional properties.

In the present paper, we phosphorylated β-Lg by dry-heating in the presence of pyrophosphate after glycation with MP through the Maillard reaction to investigate some of the

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structural and functional properties of PP−MP-β-Lg. β-Lg is tentatively considered to be involved in the binding and transportation of small hydrophobic ligands such as retinol and fatty acids, and it is categorized as a member of the lipocalin superfamily (*12*). Because it is well-known that β-Lg is a potent allergenic protein in bovine milk for human infants (*13*), we also examined the retinol-binding activity and immunogenicity of PP−MP-β-Lg.

MATERIALS AND METHODS

Materials. β-Lg was purified in the following two steps. After the crude β-Lg was isolated from raw skim milk according to the method of Armstrong et al. (*14*), it was purified by column chromatography with DEAE-cellulose (Whatman International Ltd., Maidstone, Kent, U.K.) under the following conditions: eluent, 0.05 M imidazole buffer (pH 6.7) with a 0−500 mM NaCl linear gradient; flow rate, 1.0 mL/min according to the method of Hattori et al. (*15*). The major fraction was dialyzed against Milli-Q water and then lyophilized. The purity of β-Lg was confirmed by polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (*16*); the prepared β-Lg contained variants A and B. MP and *all-trans*-retinol were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). All other reagents were of analytical grade.

Preparation of PP−MP-β-Lg. Native β-Lg (N-β-Lg) and MP (1:0.3 w/w) 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 M NaOH, followed by lyophilization. The dried sample was kept at 50 °C and 65% relative humidity (RH) for 3 days using a saturated KI solution in a desiccator according to the method given in a previous paper (*17*) 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 and 3 days, and the dry-heated samples were then dissolved in Milli-Q water. The solution was dialyzed to remove free MP and pyrophosphate for 3 days against Milli-Q water and then lyophilized.

For the preparation of β-Lg conjugated with MP (MP-β-Lg), N-β-Lg and MP (1:0.3 w/w) were dissolved in Milli-Q water at a protein concentration of 20 g/L, and the pH value of the solution was adjusted to 8.0 with 1 M NaOH, followed by lyophilization. The dried sample was kept at 50 °C (65% RH) for 3 days using a saturated KI solution in a desiccator and then dialyzed against Milli-Q water for 3 days, after which the solution was then lyophilized. For the preparation of dry-heated MP-β-Lg (DH−MP-β-Lg), MP-β-Lg before dialysis was dissolved in Milli-Q water at a protein concentration of 20 g/L, and the solution pH was adjusted to 4.0 with 1 M HCl, followed by lyophilization. The lyophilized sample was incubated at 85 °C for 3 days, after which the solution was then lyophilized. For the preparation of dry-heated β-Lg (DH-β-Lg), N-β-Lg was dissolved in Milli-Q water at a concentration of 20 g/L, and the pH was adjusted to pH 8.0 with 1 M NaOH, followed by lyophilization. The dried sample was kept at 50 °C (65% RH) for 3 days using a saturated KI solution in a desiccator and was then dissolved in Milli-Q water at a concentration of 20 g/L, and the pH was adjusted to 4.0 with 1 M HCl, followed by lyophilization. The lyophilized sample was incubated at 85 °C for 3 days, after which the solution was then lyophilized.

Determination of Sugar Content. The total sugar contents of N-, DH-, MP-, DH−MP-, and PP−MP-β-Lg were determined accroding to the phenol−sulfuric acid method (*18*). For the determination of free sugar, 2 mL of a 2 g/L sample solution was ultrafiltered through Centrisalt I (Sartorius AG-W-3400, Goettingen, Germany; molecular mass cutoff, 10000). The sugar content in the ultrafiltrate was regarded as free sugar. The sugar bound to β-Lg 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 Centrisalt I (Sartorius AG-W-3400; molecular mass cut off, 10000). The phosphorus content in the ultrafiltrate was regarded as Pi. The P content was determined by using the method of Chen et al. (*19*). The amount of phosphorus bound to proteins was estimated by the difference between the total phosphorus and Pi content.

Measurement of Solubility. Protein samples were dissolved at a protein concentration of 1 g/L in 50 mM Tris-HCl buffer (pH 7.0) and then centrifuged at 1000*g* for 15 min. The concentration of protein in the supernatant was determined by the absorbance value at 280 nm.

Electrophoresis. Native polyacrylamide gel electrophoresis (native PAGE) was performed using 14% gels in the absence of sodium dodecyl sulfate (SDS) and SDS-PAGE using 14% polyacrylamide gels under both reducing and nonreducing conditions in the presence and absence of 2-mercaptoethanol (2-ME) according to the method of Laemmli (*16*). The gels were stained in Coomassie Brilliant Blue R-250 for 1 h.

Circular Dichroism (CD) Spectra. CD spectra were measured at 195−250 nm with a Jasco J-820 spectropolarimeter (Jasco Co., Tokyo, Japan) using a cell with a 1.0 mm path length, and the digitized data were transferred to a microcomputer and processed. Samples were dissolved in 50 mM phosphate buffer (pH 7.0) at a protein concentration of 0.1 g/L. CD spectra were represented in terms of mean residue ellipticity (degrees cm^2/dmol).

Tryptophan (Trp) Fluorescence Spectra. Trp fluorescence intensity of protein samples was scanned at emissions from 300 to 400 nm excited at a wavelength of 283 nm by an FP-6600 fluorescence spectrophotometer (Jasco Co., Tokyo, Japan) at 25 °C. Each sample was dissolved in 50 mM phosphate buffer (pH 7.0) at a protein concentration of 0.1 g/L.

Differential Scanning Calorimetry (DSC). DSC was performed in a VP-DSC Microcalorimeter (MicroCal, Northampton, MA). Prior to DSC experiments, samples were dialyzed against 20 mM phosphate buffer (pH 7.4). After being filtered through a 0.22 μm filter, samples and reference solutions were properly degassed and loaded into the calorimeter. The experiments were carried out under an extra pressure of 1 atm to avoid degassing during heating. The calorimetric data were analyzed using the Origin software provided with the calorimeter. The protein concentration was 1 g/L and was heated in the calorimeter at a scan rate of 1 °C/min over a range of 30−100 °C.

Measurement of Stability of β-Lg 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). 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 5000*g* for 30 min. The soluble protein in the supernatant was measured to estimate the protein concentration of the solution by the absorbance value at 280 nm.

Measurement of Retinol-Binding Activity. The retinol-binding activity of protein samples was measured by fluorescence titration according to the method of Hattori et al. (*20*). Each sample was dissolved in PBS (0.11 M phosphate buffer, pH 7.1, containing 0.04 M NaCl and 0.02% NaN_3) at a protein concentration of 0.1 g/L, and 2 mL of the solution was put into a cuvette. Small increments (10 μL at a time) of 6.98×10^{-4} M retinol in ethanol were added to the cuvette using a micropipet. The fluorescence was measured by means of an FP-6600 fluorescence spectrophotometer (Jasco Co., Tokyo, Japan) with excitation at 330 nm and emission at 470 nm.

Immunization. An adult male JW/CSK rabbit (Charles River Japan Inc., Yokohama, Japan) was immunized subcutaneously with β-Lg emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). One month after the primary immunization, the rabbit was boosted with β-Lg emulsified in Freund's incomplete adjuvant (Difco Laboratories). Blood samples were collected 1 week after the secondary immunizations and stored at 4 °C for 24 h to form a clot. Antiserum was prepared from the sample after clot formation and verified by Ouchterlony's double-diffusion test (*21*).

Enzyme-Linked Immunosorbent Assay (ELISA). A noncompetitive ELISA was carried out according to the method of Hattori et al. (*20*). β-Lg samples dissolved in PBS 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 three times with 125 μL of PBS−Tween (PBS containing 0.5 g/L Tween 20). A 10 g/L ovalbumin/PBS solution (125 μL) was added to each well, and the

Table 1. Some Characteristics of β-Lactoglobulin (β-Lg) Evaluated

sample[a]	sugar content[b] (%)	P content[b] (%)	solubility[b] (%)
N-β-Lg	0.0 ± 0.0	0.00 ± 0.00	99.5 ± 0.5
DH-β-Lg	0.0 ± 0.0	0.00 ± 0.00	97.5 ± 0.4
MP-β-Lg	13.8 ± 0.3	0.00 ± 0.00	98.9 ± 0.5
DH–MP-β-Lg	19.4 ± 0.2	0.00 ± 0.00	98.0 ± 0.1
PP–MP-β-Lg-1d	14.8 ± 0.4	0.64 ± 0.01	98.2 ± 0.8
PP–MP-β-Lg-3d	19.8 ± 0.2	0.87 ± 0.01	99.7 ± 0.6

[a] N-β-Lg, native β-Lg; DH-β-Lg, β-Lg incubated at 50 °C (65% RH) for 3 days and then dry-heated at pH 4.0 and 85 °C for 3 days in the absence of MP and pyrophosphate; MP-β-Lg, β-Lg conjugated with MP by incubation at 50 °C (65% RH) for 3 days; DH-MP-β-Lg, MP-β-Lg dry-heated at pH 4.0 and 85 °C for 3 days in the absence of pyrophosphate; PP-MP-β-Lg, MP-β-Lg dry-heated at pH 4.0 and 85 °C for 1 and 3 days in the presence of pyrophosphate. [b] Each value is the mean with its SD ($n = 3$).

plate was incubated at 25 °C for 2 h and then washed three times. One hundred microliters of an antibody (antisera) diluted with PBS was added to each well, and the plate was incubated at 25 °C for 2 h. After three washings, 100 μL of alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (DAKO A/S, Glostrup, Denmark) 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 three times. One hundred microliters 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 (*22*). Forty microliters of 1.0 M potassium citrate, 200 μL of 0.2 M $CaCl_2$, and 240 μL of 0.2 M K_2HPO_4 were added to 2 mL of 4% protein solution, followed by the addition of 200 μL of 0.2 M $CaCl_2$ and 100 μL of 0.2 M K_2HPO_4. The addition of 200 μL of 0.2 M $CaCl_2$ and 100 μL of 0.2 M K_2HPO_4 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 by measuring the weight of the solutions. The prepared solutions were allowed to stand for 20 h at 25 °C and then centrifuged at 1000*g* for 15 min. The calcium and Pi in the supernatant were then determined (the former by using a Hitachi Z-600 atomic absorption spectrophotometer, Hitachi Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Characteristics of Phosphorylated β-Lg. β-Lg was conjugated with MP at pH 8.0 and 50 °C (65% RH) for 3 days, and MP-β-Lg was then phosphorylated by dry-heating in the presence of pyrophosphate. **Table 1** shows some characteristics of the various β-Lg samples. Although no sugar was detected in β-Lg, after incubation with MP at 50 °C (65% RH) for 3 days, the sugar content of MP-β-Lg increased to 13.8% and then further still to 19.8% by dry-heating at pH 4.0 and 85 °C for 3 days in the presence of MP and pyrophosphate. Phosphorus was not detected in β-Lg, whereas that of MP-β-Lg increased to 0.64% by dry-heating at pH 4.0 and 85 °C for 1 day in the presence of pyrophosphate (PP−MP-β-Lg-1d) and further to 0.87% by dry-heating for 3 days (PP−MP-β-Lg-3d), which was higher than that of bovine whole casein (*23*). These results suggested that glycation and phosphorylation occurred efficiently in β-Lg, as well as WPI (*11*). The solubility of β-Lg samples was measured at pH 7.0. Although solubility diminished slightly by dry-heating in the absence of MP and pyrophosphate, almost no effect of the Maillard reaction or phosphorylation on the solubility of β-Lg was observed, and even after 3 days of dry-heating in the presence of pyrophosphate, the solubility of β-Lg was 99.7%.

Figure 1A shows the native PAGE pattern of native (N-), DH-, MP-, DH−MP-, and PP−MP-β-Lg. Usually, N-β-Lg was dissociated into two bands (genetic variants of A and B). In the present study, bands A and B could be clearly observed. In the absence of MP and pyrophosphate, there were almost no changes in the mobility of bands A and B, whereas they decreased by glycation. As glycation substitutes basic amino acid side chains, it induces a slight loss of basicity and, consequently, a moderate acidification of the β-Lg. However, the mobility of MP- and DH−MP-β-Lg decreased, which might be caused by the introduction of MP to the β-Lg and the subsequent increase in their molecular mass (*11*). On the other hand, compared with MP-β-Lg, the mobility of protein increased with a rise in dry-heating time from 1 to 3 days in the presence of pyrophosphate, and that mobility increase was in agreement with the phosphorylation level (**Table 1**). These results indicated that a higher level of negatively charged phosphate groups on β-Lg produced mobility. To assess the binding type of aggregates, we performed SDS-PAGE in the absence and presence of 2-ME. It can be clearly seen from **Figure 1B** that the dry-heating induced substantial aggregation in the protein, leading to the coexistence of different populations of monomeric and multimeric β-Lg. When β-Lg was dry-heated for 3 days in the absence of MP and pyrophosphate, the intensities of the bands of aggregates increased and relative concentrations of β-Lg monomer decreased, but there were almost no changes in the mobility of β-Lg, which, however, decreased by glycation, and a different molecular mass of this protein was observed in the absence of 2-ME. This observation indicated that the molecular mass of β-Lg increased by conjugation with MP, which might explain why the mobility of β-Lg was reduced by conjugation with MP in the native PAGE (**Figure 1A**). In the absence of 2-ME, the intensities of the bands of MP-β-Lg decreased with an increase in dry-heating time from 1 to 3 days. On the other hand, in the presence of 2-ME, the intensities of the bands of MP-, DH−MP-, and PP−MP-β-Lg somewhat increased, suggesting that the formation of the polymerization between β-Lg molecules was caused by a sulfhydryl−disulfide interchange reaction through dry-heating in the absence or presence of pyrophosphate. However, some of the aggregates remained undissociated in the presence of 2-ME. These results indicated that not only disulfide bonds but also other types of bonds were formed by dry-heating. Covalent bonds other than the disulfide bonds formed in proteins by dry-heating have been discussed by some researchers (*24*, *25*), 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 (*26*). Thus, covalent bonds such as those mentioned above may be formed in β-Lg by dry-heating in the absence and presence of pyrophosphate.

Hattori et al. (*20*) have also prepared phosphorylated β-Lg by conjugation with phosphoryl oligosaccharides through the Maillard reaction. However, this phosphorylation method needs 20 days of incubation time, which is much longer than that in our method. Therefore, our method may be more useful for food applications.

Effect of Phosphorylation after Glycation on β-Lg Structure. We used CD spectroscopy to determine the impact of phosphorylation after glycation on the structural properties of

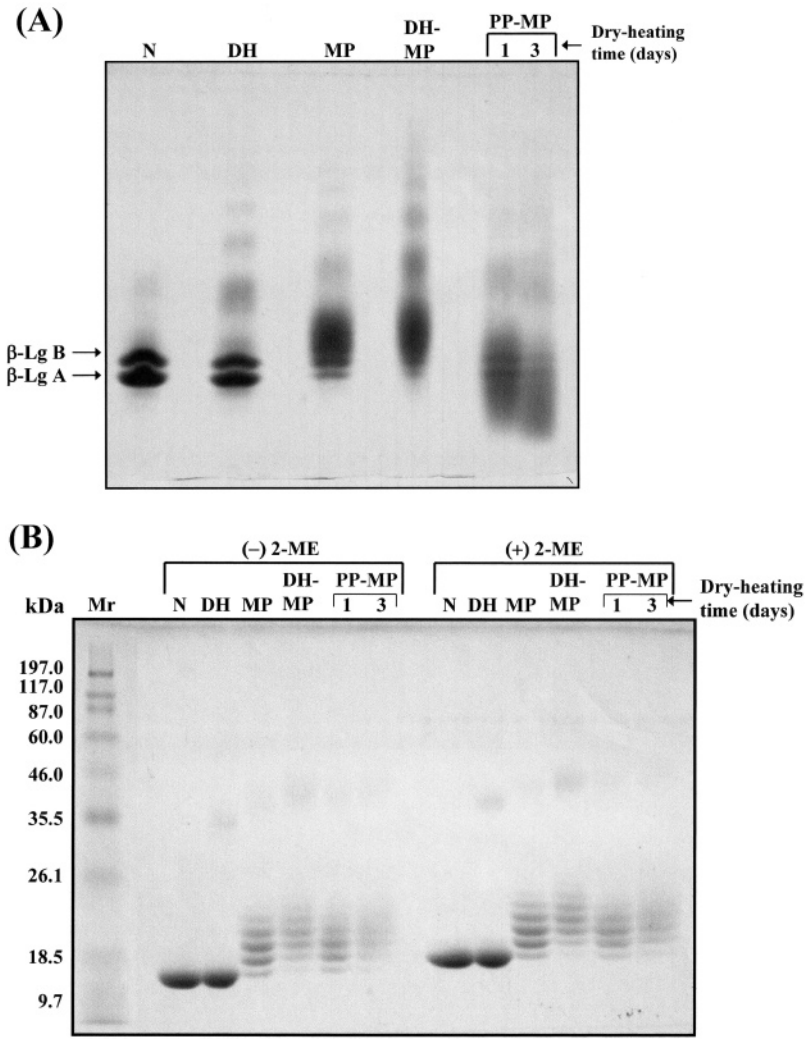

Figure 1. Electrophoretic patterns of native (N), dry-heated (DH), maltopentaose-conjugated (MP), dry-heated and maltopentaose-conjugated (DH–MP), and phosphorylated and maltopentaose-conjugated (PP–MP) β-lactoglobulin (β-Lg): (**A**) native PAGE (14% polyacrylamide gel without SDS); (**B**) SDS-PAGE (14% polyacrylamide gel with 1.7% SDS) with (+) and without (–) 5% of 2-mercaptoethanol (2-ME); Mr, marker protein.

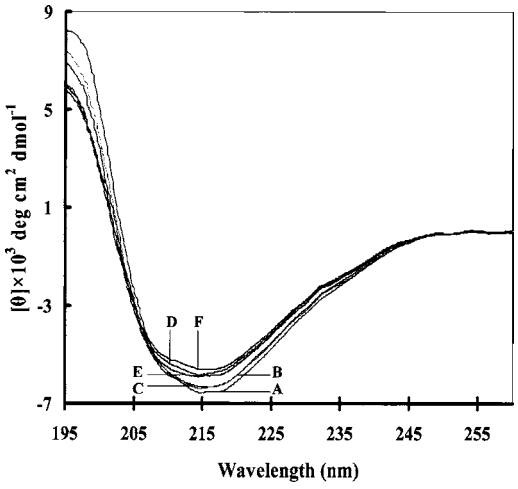

Figure 2. CD spectra of β-Lgs: (**A**) native β-Lg; (**B**) dry-heated β-Lg; (**C**) MP-conjugated β-Lg; (**D**) dry-heated MP-conjugated β-Lg; (**E**, **F**) phosphorylated and MP-conjugated β-Lg.

the protein at a secondary folding level. **Figure 2** shows the CD spectra of the β-Lg samples. The CD spectrum of N-β-Lg showed a negative maximum near 216 nm, indicating that β-Lg is rich in β-sheet structures, whereas that of β-Lg was slightly reduced by glycation and subsequent phosphorylation. These results suggested that the secondary structure of β-Lg was not significantly affected by glycation and subsequent phosphorylation.

The Trp fluorescence spectra of β-Lg are shown in **Figure 3**. The Trp fluorescence of β-Lg was slightly decreased by dry-heating alone and further decreased by glycation. From these results, it was suggested that conformational changes induced more exposure of Trp residues to solvent by dry-heating in the presence of MP (*27*), whereas the Trp fluorescence spectrum of PP−MP-β-Lg-3d was almost the same as that in DH-MP-β-Lg, suggesting that the effect of phosphorylation after glycation on the conformation around the Trp residues of β-Lg was only slight.

To investigate the thermodynamic stability of β-Lg samples, we conducted DSC experiments, and thermograms of the β-Lg samples are shown in **Figure 4**. Dimitrios et al. (*28*) reported that β-Lg contained two independent energetic domains, having different thermal stabilities. It can been seen that N-β-Lg has two domains (first and second). The denaturation temperature (T_d) of the second domain was 71.0 °C for the N-β-Lg, and only minor effects of glycation and phosphorylation on the T_d of β-Lg were observed. Dimitrios et al. (*28*) also suggested that the most stable domain (the second) contained the substrate-binding cavity. Therefore, it was considered that subtle structural

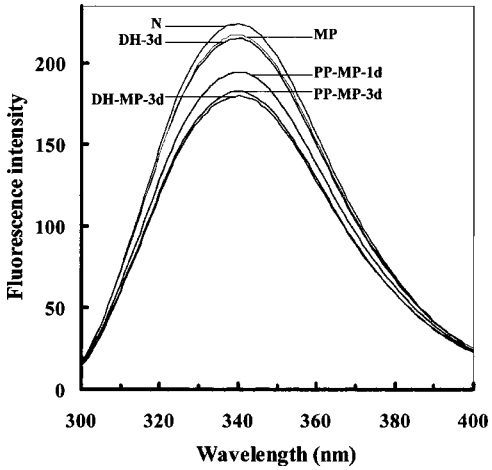

Figure 3. Tryptophan fluorescence spectra of β-Lgs. The excitation wavelength was 283 nm, and the emission was scanned from 300 to 400 nm. Fluorescence spectra of β-Lg samples were measured at 0.1 g/L in triplicate. For descriptions of **A**–**F**, see **Figure 2**.

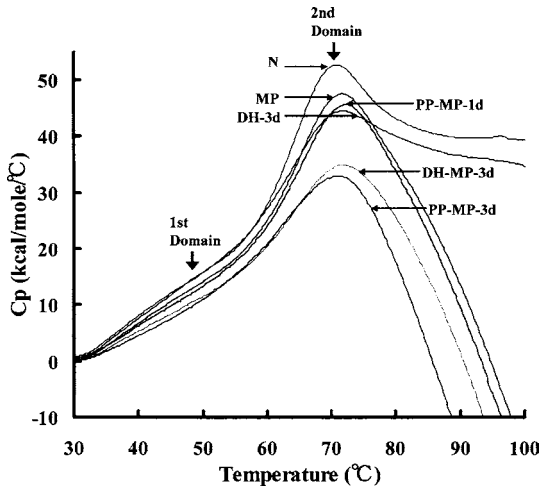

Figure 4. DSC profiles of β-Lgs. DSC scans were performed with protein solution of 1 g/L in 20 mM phosphate buffer (pH 7.4). These samples were heated in the calorimeter at a scan rate of 1 °C/min over a range of 30–100 °C. For definitions of N, DH, MP, DH–MP, and PP–MP, see **Figure 1**.

changes in the retinol-binding site of β-Lg were induced by dry-heating in the presence or absence of MP and pyrophosphate. However, the first domain was reduced by dry-heating in the presence of MP and pyrophosphate, suggesting that this domain unfolded by glycation and subsequent phosphorylation. The DSC profile of PP−MP-β-Lg-3d was similar to that of DH−MP-β-Lg, suggesting only a weak effect of phosphorylation after glycation on the structure of β-Lg molecules. These results indicated that partially unfolded conformational changes were somewhat induced in β-Lg molecules by glycation through the Maillard reaction, but that the effect of phosphorylation by dry-heating in the presence of pyrophosphate after glycation was small. In addition, considering the slight change in the CD spectra, it was suggested that changes in the gross secondary structure of β-Lg molecules by glycation and subsequent phosphorylation were small. Thus, the conformational changes of β-Lg molecule by phosphorylation after glycation were mild. When OVA was phosphorylated by dry-heating in the presence of pyrophosphate, the T_d of OVA decreased significantly (*29*). Accordingly, it was suggested that the conformational change of β-Lg induced by phosphorylation was smaller than that of

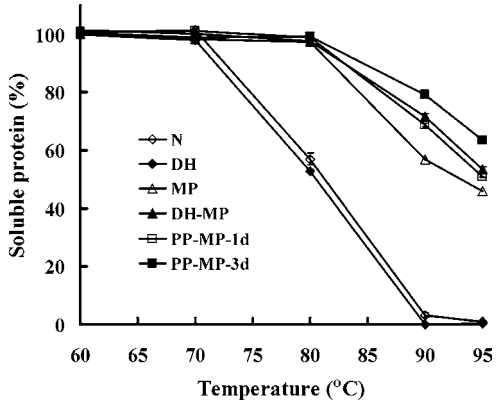

Figure 5. Stability against heat-induced insolubility of β-Lgs at various temperatures. Protein samples were 1 g/L in 50 mM Tris-HCl buffer (pH 7.0) and heated at 60–95 °C for 10 min. Each value is the mean with its SD ($n = 4$). For definitions of N, DH, MP, DH–MP, and PP–MP, see **Figure 1**.

OVA. This difference between the effects of phosphorylation on β-Lg and OVA molecules were considered to be due to the introduction level of phosphate groups to the protein moiety of OVA being higher than β-Lg. Therefore, further studies are in progress in our laboratory to examine the effect of the sugar chain of OVA on phosphorylation.

Functional Properties of Phosphorylated β-Lg. To examine the stability of β-Lg against heat-induced insolubility at pH 7.0, 1 g/L solutions of β-Lg 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 5**, soluble proteins in the N- and DH−β-Lg solutions decreased markedly at 80 °C. Furthermore, most N- and DH-β-Lgs were insolubilized at temperatures of >90 °C. In the case of MP-β-Lg, the soluble protein heated after at 90 °C was 57.0%, which was higher than those in N- and DH−β-Lgs, suggesting that the stability of β-Lg against heat-induced insolubility at pH 7.0 was considerably improved by conjugation with MP. However, under the same heat treatment temperatures (90 °C), the soluble protein in PP−MP-β-Lg-3d solution was 79.2%. These results suggested that the stability of β-Lg against heating at pH 7.0 was considerably improved by glycation and further improved by phosphorylation after glycation. The improved stability of food proteins against heating through the Maillard reaction has been reported by some researchers (*17*, *30*, *31*). Compared with DH−MP-β-Lg, the stabiliy of PP−MP-β-Lg-3d against heating at pH 7.0 was somewhat improved, suggesting that phosphate groups played a role in improving the stability of β-Lg against heat. It has been reported that the electrostatic-repulsive force is important in helping to prevent the random aggregation of denatured ovalbumin (*29*, *32*).

To assess changes in the retinol-binding activity of the β-Lg, 0.1 g/L solutions of β-Lg samples dissolved in PBS were investigated by fluorescence titration. **Figure 6** shows the retinol-binding activity of the β-Lg samples. That activity was somewhat reduced by dry-heating in the absence of MP and pyrophosphate and further reduced by glycation and subsequent phosphorylation. The PP−MP-β-Lg-3d maintained 76.5% of the retinol-binding activity of N-β-Lg, suggesting that the conformation of the retinol-binding site of β-Lg was significantly altered by glycation and subsequent phosphorylation. These results corresponded to those from DSC experiments (**Figure 4**).

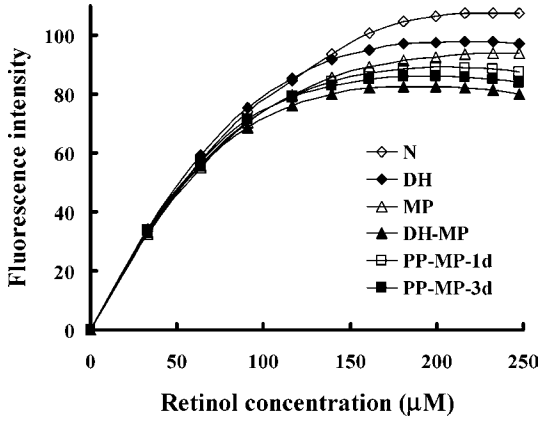

Figure 6. Retinol-binding activity of β-Lgs. Data shown are the mean value of the two determinations, with a deviation of <1%. For definitions of N, DH, MP, DH–MP, and PP–MP, see **Figure 1**.

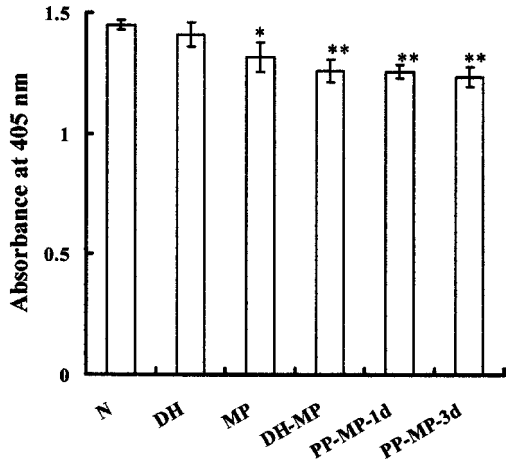

Figure 7. Immunogenicity of β-Lgs in an adult male JW/CSK rabbit. The anti-β-Lg response after the rabbit's secondary immunization was evaluated by noncompetitive ELISA, and the results are shown as ELISA values (absorbance at 405 nm). Each value is the mean with its SD ($n = 4$). * and ** indicate significant differences compared with N-β-Lg ($p < 0.05$, $p < 0.01$) as determined by Student's t test. For definitions of N, DH, MP, DH–MP, and PP–MP, see **Figure 1**.

The immunogenicity of the β-Lg samples was evaluated by measuring the reactivity of 5000-fold diluted antisera with the antigen (β-Lg) adsorbed to the solid phase of a microtitration plate by noncompetitive ELISA. As shown in **Figure 7**, the reactivity of the β-Lg was hardly affected by dry-heating in the absence of MP and pyrophosphate. However, the reactivity of the β-Lg was somewhat reduced by glycation, and the decline in reactivity corresponded to the glycation level (**Table 1**), but the reactivity was not significantly affected by phosphorylation. Anti-β-Lg antisera were believed to recognize linear epitopes rather than conformational epitopes (*20*). Therefore, this reduction in immunogenicity of the MP-β-Lg was considered to be due to shielding of the epitopes in β-Lg as a result of conjugation with MP (*20*, *33*).

The solubilization of the calcium phosphate of β-Lg was examined using the method for 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 8**, although N-, DH-, MP-, or DH−MP-β-Lg had only a slight calcium phosphate-solubilizing ability, it was enhanced by phosphorylation after glycation. In the presence of 2% protein, PP−MP-β-Lg-3d solubilized 13.4 mM Pi and 20.5 mM calcium, showing that the calcium phosphate solubilizing ability of β-Lg was efficiently enhanced by phosphorylation after glycation. Thus, PP−MP-β-Lg may be expected to enhance the absorption of calcium.

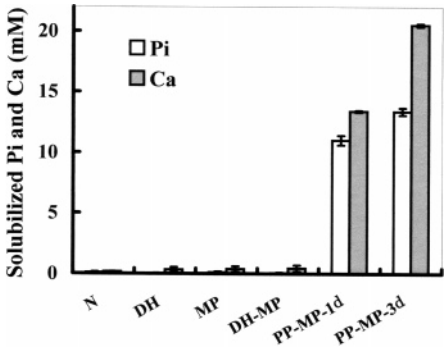

Figure 8. Calcium phosphate solubilizing ability of β-Lgs. Test solution contained 20 g/L protein, 30 mM calcium, 22 mM Pi, and 10 mM citrate, with pH adjusted to 6.7 with 1 M KOH. Each column shows the mean with its SD ($n = 3$). For definitions of N, DH, MP, DH–MP, and PP–MP, see **Figure 1**.

Summarizing the present study, we have shown that the secondary structural change of β-Lg caused by glycation and subsequent phosphorylation was small. In addition, the results of the measurement of the Trp fluorescence intensity, the DSC experiment, and the measurement of retinol-binding activity all indicated that the tertiary structure of β-Lg was somewhat changed by glycation and subsequent phosphorylation. These results from the structural study indicated that the conformational changes of the β-Lg molecule after glycation through the Maillard reaction and subsequent phosphorylation by dry-heating in the presence of pyrophosphate were mild. The functional properties of β-Lg, such as the stability against heat-induced insolubility and the calcium phosphate solubilizing ability, were improved by phosphorylation after glycation. In addition, the immunogenicity of β-Lg was somewhat decreased by glycation.

ABBREVIATIONS USED

β-Lg, β-lactoglobulin; WPI, whey protein isolate; MP, maltopentaose; PP−MP-β-Lg, MP-β-Lg phosphorylated by dry-heating in the presence of pyrophosphate; PAGE, polyacrylamide gel electrophoresis; N-β-Lg, native β-Lg; MP-β-Lg, β-Lg conjugated with maltopentaose through Maillard reaction; DH−MP-β-Lg, MP-β-Lg dry-heated in the absence of pyrophosphate; DH-β-Lg, β-Lg dry-heated in the absence of MP and pyrophosphate; Pi, inorganic phosphorus; CD, circular dichroism; Trp, tryptophan; DSC, differential scanning calorimetry; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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