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ABSTRACT: Bovine serum albumin (BSA) was phosphorylated by two methods. One is dry-heating in the presence of pyrophosphate, and the other is conjugation with maltopentaose through the Maillard reaction and subsequent dry-heating in the presence of pyrophosphate. The phosphorus content of BSA was increased to ~0.45% by dry-heating at pH 4.0 and 85 °C for 5 d in the presence of pyrophosphate, and ~0.91% by glycation and subsequent phosphorylation. The circular dichroism spectra showed that the change of secondary structure in the BSA molecule by phosphorylation was mild. However, tryptophan fluorescence intensity of BSA decreased by phosphorylation. The differential scanning calorimetry thermograms of BSA showed a disappearing of the first peak and a lowering of the second peak denaturation temperature by phosphorylation. These results indicated molten (partially unfolded) conformations of BSA formed by both phosphorylation methods. The functional properties of BSA such as heat stability and calcium phosphate solubilizing ability were improved by phosphorylation alone and further by phosphorylation after glycation. Transparent gels of BSA with relatively high water-holding capacity were obtained by phosphorylation alone, and the immunogenicity of BSA was reduced significantly by glycation and phosphorylation, respectively.

Keywords: bovine serum albumin, phosphorylation, dry-heating, structural properties, functional properties

Introduction

By-products, such as plasma from slaughtered animals or whey from cheese and casein manufacturing, are well known to be potential sources of nutritional and functional protein. Many authors have suggested the utilization of plasma proteins and whey proteins as functional ingredients in food products (Kim and others 1981; Raeker and Johnson 1995; Mandal and others 1999; Silva and Silvestre 2003). Advances in processing technology have resulted in the development of several final products, such as plasma protein isolate and whey protein isolate (WPI), which are used in food products to form gels, stabilize emulsions or forms, or as a water-holding agent (Kinsella and Whitehead 1989; Mandal and others 1999). However, they are still one of the most under-utilized by-products. The effective use of protein in food processing systems is dependent on tailoring the protein's functional characteristics to meet the complex needs of manufactured food products (Chobert 2003). Therefore, improvement of the functional properties of plasma proteins or whey proteins must maximize their effective use in the food industry.

Bovine serum albumin (BSA) is a large globular protein (66 kDa) containing 583 amino acid residues in a single chain with a known sequence (Peters 1985). The three-dimensional conformation of BSA is composed of three homologous domains (I, II, III) specific for metals and fatty acids. Each domain in turn is the product of two subdomains, which are predominantly helical, and extensively cross-linked by several disulfide bridges (Peters 1985). BSA is a major component in bovine plasma proteins, and its behavior predominantly affects functional properties of bovine plasma proteins (Mandal and others 1999). Although BSA is also a relatively minor component of whey proteins, it may contribute to their functional properties (Kinsella and Whitehead 1989). Therefore, it is important to improve functional properties of BSA in effective

utilization of plasma and whey proteins.

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 and others 1994; Kato and others 1995; Sitohy and others 1995; Vojdani and Whitaker 1996; Aoki and others 1997). However, these phosphorylation methods have posed some problems (Li and others 2003, 2004), making them very difficult to put to practical use. Li and others (2003) phosphorylated egg white protein by dry-heating in the presence of phosphate, significantly improving the heat stability, emulsifying properties, and gelling properties of egg white protein. Furthermore, the calcium phosphate solubilizing ability of egg white protein was enhanced by phosphorylation. However, WPI showed a lower phosphorylation level than egg white protein by dry-heating under the same conditions, presumably due to the lower sugar content of WPI (Li and others 2003). We then attempted to prepare phosphorylated WPI 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 and others 2005). Further study revealed that the immunogenicity of β-lactoglobulin, a major allergen in WPI, was significantly reduced by glycation with MP and subsequent phosphorylation (Enomoto and others 2007).

In the present study, we phosphorylated BSA by dry-heating in the presence of pyrophosphate with and without MP conjugation to improve their functional properties. Because BSA is well known as a major allergen in beef and milk (Fiocchi and others 1995), the effects of both phosphorylation methods on the immunogenicity of BSA also examined.

Materials and Methods

Materials

BSA (essentially globulin free), MP, and gelatin (fine powder) were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). All other reagents were of analytical grade.

Preparation of Phosphorylated BSA

Native BSA (N-BSA) was dissolved at 20 g/L in 0.1 M sodium pyrophosphate buffer at pH 4.0. The lyophilized sample was incubated at 85 °C for 5 d and then dialyzed against Milli-Q water for 3 d, after which the solution was then lyophilized (PP–BSA). N-BSA 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 d using a saturated KI solution in a desiccator according to the method given in a previous paper (Aoki and others 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 and 5 d and then dialyzed against Milli-Q water for 3 d, after which the solution was then lyophilized (PP–MP-BSA).

For the preparation of BSA conjugated with MP (MP-BSA), N-BSA and MP (1:0.3 w/w) 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 M NaOH, followed by lyophilization. The dried sample was kept at 50 °C (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 BSA (DH-BSA), BSA 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 5 d and then dialyzed against Milli-Q water for 3 d, after which the solution was then lyophilized.

Determination of Sugar Content

The total sugar contents of N-, DH-, MP-, PP–, and PP–MP-BSA were determined according to the phenol–sulfuric acid method (Dubois and others 1956). 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 cut-off 10000). The sugar content in the ultrafiltrate was regarded as free sugar. The sugar bound to BSA 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 using the method of Chen and others (1956). 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 using the method of Lowry and others

(1951).

Electrophoresis

Native polyacrylamide gel electrophoresis (native PAGE) was performed using 8.5% gels in the absence of sodium dodecyl sulfate (SDS) and SDS-PAGE using 8.5% polyacrylamide gels under both reducing and nonreducing conditions in the presence and absence 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.

Circular Dichroism (CD) Spectra

CD spectra were measured at 190–250 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. An average of five scans was recorded. 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). The protein concentration in the solution was determined using the method of Lowry and others (1951).

Tryptophan (Trp) Fluorescence Spectra

Trp fluorescence intensity (FI) of protein samples was scanned at emissions from 300 to 400 nm excited at a wavelength of 280 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. The protein concentration in the solution was determined using the method of Lowry and others (1951).

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 30–85 °C. The protein concentration in the solution was determined using the method of Lowry and others (1951).

Measurement of Stability of BSA 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. Soluble protein in the supernatant was measured to estimate the protein concentration of the solution by the absorbance value at 280 nm.

Effect of NaCl Concentration on Appearance of BSA Gel

The sample was dissolved in 20 mM phosphate buffer (pH 7.0) containing 0 to 200 mM NaCl with an interval of 25 mM NaCl to give a protein concentration of 130 g/L as determined by Kjeldahl method. The sugar contents of MP+BSA, MP-BSA, and PP–MP-BSA-1d solutions were adjusted to 19.8% by adding MP into their solutions

taking into account the sugar content in their solutions. Two hundred microliters of BSA solutions was transferred to each well of a 96-well microplate after being degassed under vacuum for 1 h, and the plate was covered with a plastic film. The microplate was then heated in an incubator at 90 °C for 1 h. The absorbance at 595 nm of solution was measured using a BioRad 550 microplate reader (BioRad Laboratories, Inc., Hercules, CA) after being heated according to the method of Kitabatake and Kinekawa (1995).

Preparation of Heat-Induced Gels for Measurement of Gelling Properties

The sample was dissolved in 20 mM phosphate buffer (pH 7.0) containing 75 mM NaCl to prepare a 130 g/L BSA solution. The BSA solution (12 mL) was degassed under vacuum for 1 h, which was transferred into a cylindrical casing tube made of polyvinylidene chloride (diameter = 13 mm, height = 100 mm), and then heated in a water bath at 90 °C for 30 min. The gels were immediately cooled to room temperature by immersion in tap water for 30 min, and allowed to stand at room temperature for 1 h. They were sectioned at 15 mm thickness and used for the measurement of mechanical properties at ambient temperature, which was carried out using a Yamaden RE-3305 rheometer (Yamaden Co., Ltd., Tokyo, Japan) equipped with a cylindrical plunger with a cross-sectional area of 2.01 cm^2 (diameter = 16 mm). The plunger descended at a rate of 0.5 mm/s until the gel ruptured. The hardness and resiliency of the gels were estimated, assuming that their surface did not change when they ruptured. The hardness and resiliency of the gels were calculated from the force–deformation curves as follows (Kang and others 1991):

hardness = maximum force (gram-force/cm^2) at rupture in the compression curve

resiliency = (area under the decompression curve/

area under the compression curve) × 100%

For measuring the water-holding capacity (WHC) of BSA gel, a 130 g/L BSA solution prepared as described above was put into a cylindrical vinyl chloride plastic casing (diameter = 13 mm, height = 50 mm) and then heated in a water bath at 90 °C for 30 min. The gels were immediately cooled to room temperature by immersion in tap water for 30 min, and allowed to stand at room temperature for 1 h. They were then sectioned at 10 mm thickness and used for measurement of WHC. WHC of gel was calculated from the formula

$$\mathrm{WHC} = (W_1/W_0) \times 100\%,$$

where W_0 was the initial gel weight and W_1 was the gel weight after being laid on five layers of filter paper (no. 2, diameter = 110 mm, Advantec Toyo Kaisha Ltd., Tokyo, Japan) at ambient temperature (25 ± 1 °C) for 1 h.

Immunization

An adult male JW/CSK rabbit (Charles River Japan Inc., Yokohama, Japan) was immunized subcutaneously with BSA emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). One month after the primary immunization, the rabbit was boosted with BSA 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 (1949).

Enzyme-Linked Immunosorbent Assay (ELISA)

A noncompetitive ELISA was carried out according to the previous paper (Enomoto and others 2007). BSA samples 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 (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 five times with 125 μL of PBS−Tween (PBS containing 0.5 g/L Tween 20). A 10 g/L gelatin/PBS solution (125 μL) was added to each well, and the plate was incubated at 25 °C for 2 h and then washed five 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 five 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 five 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 $CaCl_2$, 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 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

Statistical analysis was performed using Student's *t*-test.

Results and Discussion

Characteristics of Phosphorylated BSA

BSA was phosphorylated by dry-heating at pH 4.0 and 85 °C for 5 d in the presence of pyrophosphate (PP–BSA-5d), or conjugated with MP at pH 8.0 and 50 °C (65% RH) for 3 d through the Maillard reaction, and MP-BSA was then phosphorylated by dry-heating at pH 4.0 and 85 °C for 5 d in the presence of MP and pyrophosphate (PP–MP-BSA-5d). Table 1 shows some characteristics of the various BSA samples. Although no sugar was detected in BSA, after incubation with MP at 50 °C (65% RH) for 3 d, the sugar content of BSA increased to 11.3% and then further still to 19.8% by dry-heating at pH 4.0 and 85 °C for 5 d in the presence of MP and pyrophosphate. This suggested that glycation occurred efficiently on BSA. The phosphorous was not

301 detected in BSA, whereas that of BSA increased to 0.45% by dry-heating at pH 4.0 and
302 85 °C for 5 d in the presence of pyrophosphate. BSA was more easily phosphorylated
303 than WPI by dry-heating in the presence of pyrophosphate, but not more easily than egg
304 white protein, of which phosphorus content increased to 1.05% after phosphorylation at
305 the same conditions (Li and others 2004). The phosphorous content of MP-BSA
306 increased to 0.91% at the same conditions, which was higher than that of bovine whole
307 casein (Fox 2003). The saccharides or sugar chains in protein are phosphorylated by
308 dry-heating in the presence of phosphate (Tarelli and Wheeler 1994; Nakano and others
309 2003), suggesting that introduction of sufficient sugar may result in more efficient
310 phosphorylation of BSA by dry-heating in the presence of pyrophosphate.
311 The solubility of food protein is an important property for its application in food
312 processing. The solubility of BSA samples was measured at pH 7.0. Although the
313 solubility of BSA diminished slightly by dry-heating in the absence of MP and
314 pyrophosphate, almost no effect of glycation and phosphorylation on the solubility of
315 BSA was observed; the respective solubility of PP– and PP–MP-BSA-5d was 98.4%
316 and 97.6%.
317 Native PAGE was performed to elucidate the changes of charge in protein by both
318 phosphorylation methods. Figure 1A shows the native PAGE pattern of N-, DH-, MP-,
319 PP–, and PP–MP-BSA. There were almost no changes in the mobility of DH-BSA,
320 whereas glycation with MP decreased the mobility. As glycation modifies basic amino
321 acid side chains, it induces a slight loss of basicity and, consequently, a moderate
322 acidification of the BSA. However, the mobility of MP-BSA decreased, which might be
323 caused by the introduction of MP to the BSA and the subsequent increase of their
324 molecular mass (Li and others 2005). On the other hand, the mobility of PP– and
325 PP–MP-BSA-5d increased. These results indicated that the negatively charged

phosphate group on BSA produced mobility.

To assess the binding type of aggregates, we performed SDS-PAGE in the absence and presence of 2-ME. As shown in Figure 1B, the mobility of monomer in BSA samples treated with 2-ME was lower than those untreated with 2-ME because the three-dimensional structure was dramatically modified by the reduction of disulfide bridges and the breaking of nine loops (Peters 1985). When BSA was dry-heated for 5 d in the absence of MP and pyrophosphate, the intensities of the bands of aggregates increased, but almost no changes in the mobility of monomer was observed. However, the mobility of monomer decreased by glycation in the absence of 2-ME. This observation indicated that the molecular mass of BSA increased by conjugation with MP, which might explain why the mobility of BSA decreased by conjugation with MP in the native PAGE (Figure 1A). In the absence of 2-ME, a band was observed at the top of the gel sheet in DH-, PP–, and PP–MP-BSA-5d, whereas their intensities slightly decreased and the bands at the middle and monomer somewhat increased in the presence of 2-ME, suggesting that the formation of some of the polymerization among BSA molecules was caused by a sulfhydryl–disulfide interchange reaction through dry-heating in the absence or presence of pyrophosphate. However, a number of the aggregates remained undissociated in the presence of 2-ME, 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 and others 1989; Watanabe and others 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 in BSA by

dry-heating in the absence and presence of MP and pyrophosphate.

Effect of Phosphorylation on BSA Structure

We used CD spectroscopy to determine the respective impact of phosphorylation and phosphorylation after glycation on the structural properties of the protein at a secondary folding level. Figure 2 shows the CD spectra of the BSA samples. The CD spectrum of N-BSA showed two negative minima at 208 and 222 nm and a maximum at 190–195 nm, which are typical of the α+β class protein (Gerbanowski and others 1999). The CD spectrum of DH- and MP-BSA was close to that of N-BSA (date not shown). This spectrum was slightly changed by phosphorylation alone or glycation and subsequent phosphorylation, suggesting that the secondary structure of BSA was not significantly affected by either phosphorylation method.

The Trp fluorescence spectrum was analyzed to evaluate the conformational changes of BSA by both phosphorylation methods. As shown in Figure 3, the Trp FI of BSA decreased slightly by glycation with MP, and somewhat decreased by dry-heating alone. Furthermore, the Trp FI of BSA decreased with a slight red shift by dry-heating in the presence of pyrophosphate, and further by dry-heating in the presence of pyrophosphate after glycation. These results indicated that phosphorylation by dry-heating in the presence of pyrophosphate with and without MP induced more exposure of Trp residues to solvent (Tani and others 1995).

To investigate the thermodynamic stability of phosphorylated BSA, we conducted DSC experiments. The thermograms and denaturation temperature are shown in Figure 4 and Table 2, respectively. In the DSC profile of N-BSA, two distinct peaks (first and second) were clearly observed, suggesting that BSA contained almost no fatty acids (Michnik 2003). The denaturation temperature of first (T_1) and second (T_2) peaks were

59.4 and 78.0 °C, respectively, for the N-BSA, and only minor effects of dry-heating in the absence of MP and pyrophosphate on both T_1 and T_2 of BSA with a broadening of those peaks were observed. Although the T_1 of BSA somewhat increased to 61.8 °C by glycation, its DSC profile was also bimodal. However, the first peak of BSA completely disappeared by both phosphorylation methods. Furthermore, the T_2 decreased by both phosphorylation methods, and those of PP– and PP–MP-BSA-5d were 74.8 and 72.8 °C, respectively. Michnik (2003) assumed that the carboxyl-terminal fragment consisting of domain III and a large part of domain II melted at lower temperatures, while the second amino-terminal fragment consisting of domain I and a small part of domain II melted at higher temperature. Therefore, it was assumed that the C-terminal containing those domains in BSA collapsed and the rest of the molecule somewhat unfolded due to the introduction of electrostatic-repulsive force of phosphate groups in the BSA molecule. However, given the slight change in the CD spectra, it was suggested that the changes in gross secondary structure of BSA molecules by both phosphorylation methods were mild and that molten (partially unfolded) conformations were formed by phosphorylation.

Functional Properties of Phosphorylated BSA

To examine the stability of BSA against heat-induced insolubility at pH 7.0, 1 g/L solutions of BSA 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 protein in the N- and DH-BSA solutions decreased markedly as heating temperature increased to >70 °C, and then declined to ~8.8% for N-BSA and ~5.9% for DH-BSA by heating at 80 °C. The soluble protein after heating at 80 °C was 34.4% for MP-BSA and 26.9% for PP–BSA-5d, respectively, which were higher than

401 those in N- and DH-BSA, but most of the proteins were insolubilized at temperatures of
402 >90 °C. However, in the case of PP–MP-BSA-5d, 98.7% of protein remained soluble
403 after heat treatment at 80 °C. These results suggested that the stability of BSA against
404 heating at pH 7.0 was somewhat improved by glycation alone and phosphorylation
405 alone respectively, and further improved by phosphorylation after glycation. The
406 improved heat stability of food proteins through the Maillard reaction has been reported
407 by some investigators (Kato and others 1989; Aoki and others 1999; Matsudomi and
408 others 2002). Kato and others (1995) reported that the heat stability of ovalbumin was
409 more improved by conjugation with glucose-6-phosphate than by conjugation with
410 glucose, suggesting phosphate groups played an important role in improving the
411 stability of ovalbumin against heat. It has been reported that the electrostatic-repulsive
412 force is important in helping to prevent the random aggregation of denatured food
413 proteins (Kitabatake and others 1988; Li and others 2005; Enomoto and others 2007).
414 We measured some properties of heat-induced BSA gels to determine whether they
415 were improved by both phosphorylation methods. First, we examined the effect of NaCl
416 concentration on the appearance of BSA gels at 595 nm. The N- or MP+BSA gels were
417 prepared to compare with PP–BSA-5d, or MP- and PP–MP-BSA gels, respectively. As
418 shown in Figure 6, the turbidity of N- and MP+N-BSA gels increased with an increase
419 in NaCl concentration. Although gel turbidity was somewhat reduced by glycation with
420 MP, the gels remained opaque at a NaCl concentration of >100 mM. On the other hand,
421 the turbidity of PP–BSA-5d and PP–MP-BSA gels was much lower than that of N-,
422 MP+N-, and MP-BSA gels, becoming very transparent under visual inspection at a
423 NaCl concentration area measured in this study (data not shown). Thus, a NaCl
424 concentration of 75 mM was used in subsequent experiments for the measurement of
425 gel properties such as hardness, resiliency and WHC of BSA gels. As shown in Table 3,

426 the hardness and resiliency of PP–BSA-5d gel were significantly lower than those of
427 N-BSA. Compared with MP+BSA, the significant decrease of the hardness and increase
428 of the resiliency in PP–MP-BSA-5d gel was observed, although those of MP-BSA and
429 PP–MP-BSA-1d gels did not significantly change. The WHC of BSA gels was
430 increased significantly by phosphorylation alone and even more by phosphorylation
431 after glycation. The turbidity of BSA solution (75 mM NaCl) before heating and gels
432 after heating are shown in Table 3. Before heating, although the turbidity of all samples
433 was low (as expected), that of N-, MP+N-, and MP-BSA gels increased after heating at
434 90 °C for 1 h. However, the turbidity of PP–BSA-5d and PP–MP-BSA was much lower
435 than that of N-, MP+N-, and MP-BSA. Furthermore, it seems that transparent
436 heat-induced PP–BSA-5d and PP–MP-BSA gels could be produced at a relatively
437 higher NaCl concentration. The improved WHC and increased transparency of these
438 gels in the present study were considered to be due to the construction of a uniform
439 network (Woodward and Cotterill 1986; Yasuda and others 1986). Interestingly,
440 although PP–MP-BSA-5d contained more phosphorus than PP–MP-BSA-1d (0.43%),
441 its heat-induced gel was softer than that of PP–MP-BSA-1d at 75 mM NaCl, most likely
442 due to too strong electrostatic repulsive force of phosphate groups (Hatta and others
443 1986).

444 The immunogenicity of BSA samples was evaluated by measuring the reactivity of
445 1000-fold diluted antisera with the antigen (BSA) adsorbed to the solid phase of a
446 microtitration plate by noncompetitive ELISA. As shown in Figure 7, the reactivity of
447 the BSA was hardly affected by dry-heating in the absence of MP and pyrophosphate.
448 However, the reactivity of the BSA was reduced significantly by glycation and
449 phosphorylation, respectively, further reduced by phosphorylation after glycation. IgE
450 recognizes specific conformational and linear molecular structures on allergenic

proteins. Restani and others (2004) suggested the presence of both conformational and linear epitopes on BSA molecule. Thus, these reductions in immunogenicity of the PP–MP-BSA-5d were considered to be due to shielding of the linear epitopes by conjugation with MP (Enomoto and others 2007) and unfolding of the conformational epitopes by the electrostatic-repulsive force of the introduced phosphate groups. Therefore, the glycation and subsequent phosphorylation were considered to be effective for reducing the immunogenicity of BSA.

The solubilization of the calcium phosphate of BSA 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. As shown in Figure 8, although N-, DH-, and MP-BSA had only a slight calcium phosphate solubilizing ability, it was enhanced by phosphorylation. In the presence of 2% protein, PP–BSA-5d solubilized 3.1 mM Pi and 3.9 mM Ca. Furthermore, PP–MP-BSA-5d solubilized 12.2 mM Pi and 21.3 mM Ca, showing that the calcium phosphate solubilizing ability of BSA was efficiently enhanced in correspondence with the phosphorylation level (Table 1). Thus, both phosphorylated BSA, especially PP–MP-BSA-5d, may be expected to enhance the absorption of calcium.

Conclusions

We have shown that BSA was successively phosphorylated by dry-heating in the presence of pyrophosphate with and without MP conjugation. Although the secondary structural change of BSA was small, the results of the measurement of Trp FI and DSC experiments indicated that the tertiary structural change of BSA was significant by both phosphorylation methods, suggesting that molten (partially unfolded) conformations of BSA were formed by phosphorylation. The functional properties of BSA, such as heat

476 stability and calcium phosphate solubilizing ability, were improved by phosphorylation
477 alone and further by phosphorylation after glycation. Transparent gels of BSA with
478 relatively high WHC were obtained by phosphorylation, and the immunogenicity of
479 BSA was reduced significantly by glycation and phosphorylation, respectively.
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