Note

Changes in Solubility and Thermal Stability of Chicken Myofibrillar Protein by Glycosylation

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Myofibrillar protein prepared from chicken breast muscle was incubated with several concentrations of glucose or maltose for 6 h at 60°C and 35% relative humidity in order to obtain glycosylated chicken protein. When the ratio of the weights of the myofibrillar protein and glucose or maltose exceeded 1:6 or 1:3-5, respectively, the solubility of each type of glycosylated chicken protein in 0.1 M NaCl solution exceeded by about 60%, although the myofibrillar protein was insoluble in a low ionic strength solution. Solubilization of chicken myofibrillar protein required a small quantity of glucose compared with carp myofibrillar protein. Neither sample underwent denaturation when held at 50°C for 2 h.

Keywords: chicken, myofibrillar protein, glycosylation, Maillard reaction, solubility, thermal stability

Abbreviations: RH, relative humidity; TCA, trichloroacetic acid

Introduction

Although chicken is cheap and contains nutritious proteins and lipids compared to pork and beef (Youling, 2000), the diversity of its processed food is poorer than that of pork. Therefore, it may be beneficial to develop new functionality of chicken as a material for processed food.

Saeki and colleagues have reported improved functional properties of fish and shellfish myofibrillar proteins by glycosylation using the Maillard reaction (Saeki, 1997; Saeki and Inoue, 1997; Saeki and Tanabe, 1999; Fujiwara *et al.*, 1998; Katayama *et al.*, 2002). Carp and scallop myofibrillar proteins become solubilized in a low ionic strength medium by reaction with glucose and ribose (Saeki and Inoue, 1997; Saeki and Tanabe, 1999; Katayama *et al.*, 2002). Both the emulsifying properties and thermal stability of carp myofibrillar proteins can also be enhanced by conjugation with dextran (Fujiwara *et al.*, 1998). Functional improvement of this protein occurred when lyophilized myofibrillar proteins

were reacted and conjugated with reducing sugars through the early stage of the Maillard reaction.

However, the fish muscular protein is thermally less stable than that of other animals (Connell, 1961). Thus, a comparatively large amount of sugar has to be used for glycosylation to prevent denaturation of the fish meat protein (Boye *et al.*, 1996). In fact, Saeki and Inoue used as much as nine-fold glucose for the weight of proteins to make myofibrillar proteins of a carp solubilize in low ionic strength medium by glycosylation (Saeki and Inoue, 1997). In contrast, muscular proteins of homothermal animals have superior thermal stability (Connell, 1961). Thus, functional alteration in the conjugation of chicken myofibrillar proteins is expected to occur with less sugar than that required for fish.

To expand the availability of neoglycoprotein synthesized using chicken myofibril as a new food material, in this study, we followed the solubility changes of low and high ionic strength media of chicken myofibrillar protein during the reaction with glucose as a monosaccharide or maltose as a disaccharide in relation to the progress of the Maillard reaction. The smallest amount of both sugars showing solubility

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of 60% or more in low ionic strength medium was examined and this minimum amount was used to investigate thermal stability.

Materials and Methods

Materials and chemicals Breast meat was removed from chickens immediately after slaughter. The glycosylated human serum calibration kit was purchased from Japan Roche Diagnostic, Inc. (Tokyo, Japan). The other chemicals used were of reagent grade and obtained from Nacalai Tesque, Inc. (Kyoto, Japan), or Wako Pure Chemicals Industries, Ltd. (Osaka, Japan).

Myofibril preparation The chicken myofibril fraction was prepared using a modification of the method of Saeki (1997). Briefly, chicken breast meat was ground with a food chopper and suspended in 10 volumes of 0.1 M sodium phosphate buffer (pH 7.5); the supernatant was removed by decantation. This step was repeated twice. The washed and ground meat was homogenized with a homogenizer (AM-6; Nissei Co., Ltd., Tokyo, Japan) in 10 volumes (based on initial muscle weight) of 0.1 M sodium phosphate buffer (pH 7.5) for 0.5 min at 10,000 rpm. This step was repeated 3 or more times. After filtration through cotton gauze to remove impurities, 20% Triton X-100 solution was added to the obtained filtrate to a final concentration of 0.5% and stirred gently using a stirrer for 3 min. After standing in 10 min, the filtrate was centrifuged at 7,000 g for 10 min to collect myofibrils. The precipitate was resuspended in 50 mM NaCl and centrifuged. This procedure was carried out 3 or more times. The purified chicken myofibril was obtained by filtering through a cotton gauze. All preparation steps were carried out on ice.

Glycosylation of myofibrillar proteins Glycosylation of myofibrillar proteins was performed using a modification of the method of Saeki (1997). Briefly, myofibril suspended in 50 mM NaCl was mixed with glucose at weight ratios of 1:4, 1:5, 1:6, 1:9, and 1:18, and with maltose at weight ratios of 1:3, 1:4, 1:5, and 1:6. After final protein concentrations were adjusted to 6.0 mg/mL, 5 mL of the myofibril-glucose and myofibril-maltose solution were each placed in a test tube (diameter: 16 mm), frozen at -80°C , and immediately lyophilized using a freeze-dryer (FD-1; Tokyo Rika Co. Ltd., Tokyo, Japan), destroying the sarcomere and exposing myofibrillar proteins. The lyophilization was stopped when the sample temperature reached $15 - 18^{\circ}\text{C}$. The lyophilized protein powder was immediately stored at -40°C and used within 30 days of preparation.

To react protein with glucose and maltose through the Maillard reaction, both lyophilized protein powders were incubated at 60°C and 35% relative humidity (RH) for 0 – 6

h. An incubator/humidity cabinet (L-200A; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) was used for controlling the temperature and RH. In this study, myofibril alone (without sugar) was also lyophilized and incubated under the same conditions as the proteins-sugar mixtures containing glucose and maltose.

Determination of available lysine and fructosamine

Available lysine and fructosamine assays were carried out to evaluate the progress of the Maillard reaction between myofibrillar proteins and glucose or maltose. Myofibrillar proteins that reacted with glucose and maltose were dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl with a high speed blender (Ultra-turrax T25-S1; IKA-Labortechnik, Staufen, Germany), precipitated with 7.5% trichloroacetic acid (TCA) (at final concentration) at room temperature for 30 min. After removal of the Tris buffer, unreacted sugars, and TCA by decantation, the precipitated protein was redissolved in 50 mM sodium phosphate buffer (pH 9.5) containing 2% sodium dodecyl sulfate. Available lysine content was determined by spectrophotometric analysis using *o*-phthalaldehyde and *N*-acetyl-L-cysteine (Hernandez and Alvarez-Coque, 1992).

Myofibrillar proteins that reacted with glucose and maltose were dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and dialyzed against the same buffer at 4°C for 16 h to remove unreacted sugars, and then fructosamine was assayed by the method of Johnson *et al.* (1983). Glycosylated human serum was used as a standard for determining fructosamine content which was regarded as the ketoamine content. The available lysine and fructosamine assays were performed within 48 h after sample preparation.

Solubility of glycosylated myofibrillar proteins Solubility of glycosylated myofibrillar proteins was measured according to a modified method of Saeki and Inoue (1997). After incubation for glycosylation, the protein powders were immediately mixed with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 or 0.5 M NaCl at 1.5 mg/mL of the final protein concentration with the high speed blender at 10,000 rpm for 0.5 min. After homogenization was repeated, the protein solution was centrifuged at 15,000 g for 30 min at 4°C . Proteins in solution before and after centrifugation were precipitated with 7.5% TCA (at final concentration) at room temperature for 30 min. After removal of Tris buffer, unreacted sugars, and TCA by decantation, the precipitated protein was redissolved in 1 M NaOH. The total solubility of the myofibrillar proteins was expressed as percent protein concentration in the supernatant with respect to that of the total protein solution before centrifugation.

Thermal stability Thermal stability was followed by monitoring changes in solubility of the protein solution dur-

ing heating according to a modified method of Fujiwara *et al.* (1998). As Murphy *et al.* (1998) reported that thermal denaturation of chicken myofibrillar protein occurs at 53°C, the incubation temperature was set at 50°C to examine the effect of the temperature of this denaturation area on solubility. Lyophilized myofibrillar proteins mixed with glucose and maltose at a weight ratio of 1:6 and 1:4, respectively, were kept at 60°C and 35% RH for 6 h. After incubation for glycosylation, the protein powders were immediately mixed with 40 mM Tris-malate buffer (pH 7.5) containing 0.1 or 0.5 M NaCl at 2 mg/mL of the final protein concentration with the high speed blender at 10,000 rpm for 0.5 min twice (total of 1 min) and dialyzed against the same NaCl solution at 4°C for 16 h. Unreacted glucose and maltose were removed at this step. The dialyzate stood at 50°C for 2 h and then was centrifuged at 15,000 *g* for 30 min at 4°C. Thermal stability was expressed as a percent of protein concentration in the supernatant with respect to that of the dialyzate before the 2-h

incubation at 50°C.

Protein determination Protein concentration was determined by the method of Lowry *et al.* (1951) using ovalbumin as the standard in the case of the available lysine assay, and the Biuret method (Gornall *et al.*, 1949), using bovine serum albumin as the standard, was used for the other assays.

Statistical analyses All experiments were carried out with samples from at least three different lots of glycosylation. Values indicated in the figures are the means of at least 3 determinations, and the error bars indicate standard deviation. Student's *t*-test (Gosset, 1908) was used to determine the significance of differences among samples.

Results and Discussion

Reaction between myofibrillar proteins and glucose or maltose Figure 1 shows the change in available lysine and fructosamine contents in lyophilized myofibrillar proteins during reaction with glucose or maltose at 60°C and 35%

Fig. 1. Time-course changes in the concentrations of available lysine and fructosamine in myofibrillar proteins during the reaction with glucose and maltose at different mixing ratios of the myofibril and sugars.

Lyophilized myofibrillar protein-glucose mixture at weight ratios of 1:4 (—■—), 1:5 (—◆—), 1:6 (—▲—), 1:9 (—●—), and 1:18 (··—×—··); lyophilized myofibrillar protein-maltose mixture at weight ratios of 1:3 (—◇—), 1:4 (—□—), 1:5 (—△—), and 1:6 (··—○—··) were held under 60°C and 35% RH for 0, 1, 3, and 6 h, respectively. The amounts of available lysine in the glycosylated myofibrillar protein with glucose (A) and maltose (C) were recorded as the reaction progressed. Simultaneously, the quantities of fructosamine in the glycosylated myofibrillar protein with glucose (B) and maltose (D) were measured.

RH. The amount of available lysine before incubation was 0.084 ± 0.006 g/g of protein ($n = 21$). The available lysine content in protein glycosylated with glucose (Figure 1A) decreased with reaction time to the average of 24.6% after 6 h, and the fructosamine content (Figure 1B) rose simultaneously to reach to the mean of $427.3 \mu\text{mol/g}$ of protein. Although some values showed significant differences with each other ($p < 0.05$), the progress of change in available lysine or fructosamine content was similar. When lyophilized myofibrillar proteins reacted with maltose, the available lysine content (Figure 1C) decreased to the average value of 68.0%; there was no significant difference among all four proteins glycosylated with maltose. The fructosamine content (Figure 1D) rose to the mean of $230.9 \mu\text{mol/g}$ protein after the 6-h incubation. Although some values showed significant differences from each other ($p < 0.05$), the progress of change in fructosamine content was similar. As for both proteins glycosylated with glucose and maltose, the molar quantity of fructosamine produced was almost equal to that of the available lysine reduced over 6 h. When the lyophilized protein without sugar was incubated under the same conditions, the available lysine content remained unchanged and no fructosamine was

produced. Additionally, patterns of SDS-polyacrylamide gel electrophoresis with 1% 2-mercaptoethanol showed that myosin and actin mobility decreased with the reaction (data not shown). These results indicated that glucose or maltose react with the lysine residues in myofibrillar proteins. Glucose was then combined with a protein more promptly compared with maltose to generate fructosamine. This high reactivity is considered to be based on the molar quantity of the carbonyl group in glucose being twice as much as that in the same weight of maltose. This consideration was supported by the report that reactivity is extremely low in dextran—a polysaccharide (Fujiwara *et al.*, 1998).

Proteins solubilized in low and high ionic strength media with glycosylation Changes of the solubility of proteins glycosylated with glucose and maltose in 0.1 and 0.5 M NaCl are shown in Figures 2 and 3, respectively. The solubility of myofibrillar proteins in 0.5 M NaCl was about 80%, which showed almost no change during 6 h of the Maillard reaction when glucose was used at mixing ratios of 1:18, 1:9 and 1:6, while it decreased during the glycosylation process to less than 60% when glucose was used at mixing ratios of 1:5 and 1:4 (Figure 2). Saeki and Inoue (1997) also observed

Fig. 2. Changes in the solubility of myofibrillar protein reacted with glucose. Lyophilized myofibrillar protein-glucose mixtures at weight ratios of 1:18 (A), 1:9 (B), 1:6 (C), 1:5 (D), and 1:4 (E) were held under 60°C and 35% RH for 0, 1, 3 and 6 h, respectively; solubility of the myofibrillar protein in 0.1 M (◆) and 0.5 M (■) NaCl solution (pH7.5) was measured.

Fig. 3. Changes in the solubility of myofibrillar protein reacted with maltose. Lyophilized myofibrillar protein-maltose mixtures at weight ratios of 1:6 (A), 1:5 (B), 1:4 (C), and 1:3 (D) were held under 60°C and 35% RH for 0, 1, 3, and 6 h, respectively; solubility of the myofibrillar protein in 0.1 M (◆) and 0.5 M (■) NaCl solution (pH 7.5) was measured.

such decreases in solubility and suggested that the cause depended on heat denaturation of the myofibrillar proteins. Protein is known to be stabilized in the presence of glucose (Boye *et al.*, 1996; Chen and Chang, 1998; Christ *et al.*, 2005). As shown in Figures 1A and 1B, the progress of the Maillard reaction were similar among the myofibrillar protein-glucose systems, indicating that the loss of protein solubility with a decrease in glucose concentration in protein-glucose mixture (Figures 2D and 2E) seems to be caused by a shortage of glucose as a thermal protectant. On the other hand, the solubility in 0.1 M NaCl increased markedly with glycosylation, reaching 88.0 ± 2.9 , 83.1 ± 4.4 , 65.3 ± 12.7 , 59.0 ± 12.4 , and $52.4 \pm 1.4\%$ ($n = 3$) after 6 h at mixing ratios of 1:18, 1:9, 1:6, 1:5, and 1:4, respectively. During glycosylation with glucose content 6 times that of the protein, protein denaturation in 0.5 M NaCl hardly occurred and the solubility of the protein in 0.1 M NaCl reached more than 60%, suggesting that the minimum mixing ratio of myofibrillar protein to glucose was 1:6.

As for maltose (Figure 3), the solubility of myofibrillar

proteins in 0.5 M NaCl was almost unchanged during 6 h of the Maillard reaction at the mixing ratios from 1:6 to 1:3 of myofibrillar protein to maltose. Izutsu *et al.* (2004) reported that maltose stabilizes the protein better than glucose. Thus, for maltose, a rapid decrease in solubility seen for glucose (Figures 2D and 2E) is not expected by this protective effect. The solubility in 0.1 M NaCl at the mixing ratio of 1:6 reached about 80%, while other samples (at 1:3-5) attained about 60% after 6 h.

Figures 1A and 2 indicate that the solubility improvement of chicken myofibrillar proteins occurs when about 60% of available lysine reacts with glucose since the solubility improvement in low ionic strength medium occurred after 3 h of incubation. On the other hand, about 30% loss of available lysine is required to achieve solubility improvement with maltose, for which 6-h incubation was indispensable (Figures 1C and 3). As reported by Babij *et al.* (1991), about 200 lysine residues exist in a molecule of myosin heavy chain, and about 120 glucose and 60 maltose molecules were considered to be conjugated with a myosin heavy chain

when the solubility improvement occurred. One molecule of glucose has 5 hydroxy groups, while 8 hydroxy groups exist in maltose, including a hydroxyl group connected with an anomeric carbon. Thus, the total number of hydroxy groups in these sugar molecules is 600 and 480, respectively. However, the number of hydroxy groups connected by the anomeric carbon for combining with lysine was increased by ring-opening, resulting in invariability of the total number of hydroxy groups. Although there is a small difference between the numbers of hydroxy groups per a myosin heavy chain, we assumed that about 500 hydroxy groups are conjugated with one myosin heavy chain to increase the hydration with water allowing for solubility of myofibrillar protein. Katayama *et al.* (2002) suggested the dissociation of myosin and actin due to a structural impediment on the actin-binding site in subfragment 1 by the attached glucose as one cause of solubility improvement. Moreover, the ϵ -amino group of lysine residues has been shown to play an important role in the self-association of the myosin rod filaments (McLachlan and Karn, 1982; Kato and Konno, 2000). Katayama and Saeki (2007) also suggested that water solubilization is caused by the loss of the filament-forming ability by glycosylation. Of course, such a steric hindrance must also take part in solubility improvement.

Thermal stability Unglycosylated myofibrillar protein and those glycosylated with 6 times of glucose and 4 times of maltose were dissolved in 0.1 and 0.5 M NaCl solutions (pH 7.5) and then heated at 50°C for 2 h. Although there were not conspicuous differences between the solubility of myofibrillar proteins glycosylated with 3-5 times of maltose in 0.1 M NaCl (Figure 3), the solubility in 0.1 M NaCl was the highest at the mixing ratio of 1:4 for myofibrillar protein to maltose when the reaction time was extended by 24 h (Nishimura *et al.* in press). Accordingly, the mixing ratio of 1:4 was chosen to examine thermal stability. Figure 4 shows the solubility of glycosylated and unglycosylated myofibrillar protein before and after heating. Unglycosylated myofibrillar protein in 0.5 M NaCl decreased significantly from 66% to 13% ($p < 0.01$) during heat treatment. However, for myofibrillar protein containing glucose and maltose in 0.1 M and 0.5 M NaCl, a significant decrease in solubility was not observed at the start or end of the 2-h heating. These results indicate that the thermal stability of chicken myofibrillar protein is improved by conjugation with both sugars and that the mixing ratios of 1:6 and 1:4 for myofibrillar proteins to glucose and maltose, respectively, is enough to acquire thermal stability in 0.1 and 0.5 M NaCl. This improvement in the thermal stability of myofibrillar protein might be caused by suppressing thermal

Fig. 4. Effects of glycosylation with glucose or maltose on the thermal stability of myofibrillar protein.

Group A shows the solubility of each type of glycosylated myofibrillar protein before 2-h incubation at 50°C. Group B shows the solubility of each type of glycosylated myofibrillar protein after incubation. Myofibrillar protein in 0.5 M NaCl solution (a and a'), myofibrillar protein glycosylated with 6 × glucose (b and b'), and myofibrillar protein glycosylated with 4 × maltose (c and c') in 0.5 M NaCl solution. The same neoglycoproteins including glucose (d and d') and maltose (e and e') in 0.1 M NaCl solution. They were incubated for 2 h at 50°C, and the solubility before and after 2-h incubation at 50°C for each type of sample was calculated. ** $p < 0.01$.

aggregation of myosin by attaching sugars and the increase of hydration with water due to the presence of a sufficient amount of hydroxy groups.

Chicken myofibrillar protein glycosylated with glucose and maltose at mixing ratios of 1:6 and 1:4, respectively, through 6-h incubation at 60°C and 35% RH improved both the solubility and thermal stability. In particular, the functional change in chicken myofibrillar protein did not require the large amount of glucose which carp myofibrillar protein demanded. Takeda *et al.* (2007) already examined the feasibility of industrial application and indicated the strong potential of protein glycosylation for use with spawned-out chum salmon. These results indicate that glycosylated chicken myofibril could be an available new food material.

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