Improved Solubility of Carp Myofibrillar Proteins in Low Ionic Strength Medium by Glycosylation

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Carp myofibrillar proteins were glycosylated with glucose through the Maillard reaction (the early stage), and their solubility in various ionic strength media was investigated. To react with glucose, lyophilized myofibrils mixed with glucose (at weight ratio of 1:9) were kept at 40 °C and 65% relative humidity for 0−48 h. By glycosylation, myosin and actin became solubilized in low ionic strength media. The total solubility of myofibrillar proteins in 0.01−0.16 M NaCl increased with the progress of glycosylation and was almost equal to the solubility of 0.5 M NaCl. When 17% lysine residue in myofibrillar proteins was reacted with glucose, 61% myosin and 82% actin were solubilized in 0.1 M NaCl. These results indicate that water-soluble myofibrillar proteins can be prepared by glycosylation with the Maillard reaction.

Keywords: *Fish; myofibrillar proteins; neoglycoprotein; glycosylation; Maillard reaction; solubility*

INTRODUCTION

Fish meat is an abundant protein resource and is widely used as a material for processed foods. Functional properties of fish meat such as gel-forming ability, emulsifying property, and water-holding ability are related to solubilization of myofibrillar proteins in salt solutions (Suzuki, 1981; Regenstein et al., 1983; Akahane et al., 1984). Many researchers have investigated the relation between the functional properties and the solubility of myofibrillar proteins (Niwa, 1992). For instance, solubilization of myosin, the major protein of meat, in high ionic strength solutions (>0.3) is required for strong gelation of fish meat. The solubility of myosin in a high ionic strength medium is often used as a sensitive indicator for evaluating protein denaturation (Koseki et al., 1993; Careche and Tejada, 1994). On the other hand, attempts have been made to solubilize fish meat in water by proteolytic enzymes and by acid hydrolysis for human consumption, animal feed, and liquid fertilizers (Sugii and Kinumaki, 1973; Archer et al., 1973; Miyake, 1982; Piggot and Tucker, 1990). However, production of peptides occurring from protein hydrolysis is often responsible for a bitter and unacceptable taste. Stefansson and Hultin (1994) have also reported that cod myofibrillar proteins such as myosin, actin, and tropomyosin are solubilized in water at neutral pH if the final ionic strength is approximately 0.0003 or less. However, this property is not feasible for food production because the ionic strength of food cannot be adjusted to such a low ionic strength. Thus the authors thought that a better procedure for dissolving fish myofibrillar proteins in solutions of low ionic strength of about 0.05−0.2 M would contribute to more effective use of fish meat by the food industry.

Recently, it has been reported that glycosylation of proteins by the Maillard reaction is an effective method for improving the functional properties of proteins (Kato et al., 1981, 1993a,b; Aoki et al., 1994). Saeki (1997) reported that the emulsifying properties of carp myofibrillar proteins were improved when glycosylation by the Maillard reaction was carried out at an early stage. In this study, the change in the solubility of myofibrillar proteins in low ionic strength media as affected by the glycosylation were investigated.

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MATERIALS AND METHODS

Materials. A cultured live carp (*Cyprinus carpio*) was obtained at a local fish market. A fructosamine test calibration kit (glycosylated human serum) was obtained from Japan Roche Co. Ltd. All other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Myofibrils. Carp ordinary muscle was ground with a meat chopper and was washed in 3 vol of 50 mM NaCl and 0.5% Triton X-100 for 10 min. After removing the supernatant by decantation, the washed ground meat was homogenized using a homogenizer (model AM-6, Nissei Co. Ltd., Tokyo, Japan) in 8 vol (based on initial muscle weight) of 50 mM NaCl and 0.5% Triton X-100 for 2 min at 20 000 rpm. After filtration through cotton gauze to remove connective tissues, the homogenate was centrifuged at 8000*g* for 10 min to collect myofibrils. The precipitate was resuspended in the 50 mM NaCl and centrifuged four times. The purified myofibrils thus obtained were filtered through a nylon cloth. All preparation steps were carried out below 8 °C. The protein concentration was determined by the biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

Glycosylation of Myofibrillar Proteins. Glucose at a final concentration of 0.3 M was added to the myofibrils, and the protein concentration was adjusted to 6.0 mg/mL. A 5 mL amount of the mixture was placed in a test tube (diameter: 16 mm) and frozen at −40 °C and immediately lyophilized using a freeze-dryer (FDU-506, Tokyo Rika Co. Ltd., Tokyo, Japan). The lyophilization was stopped when the sample temperature reached 15−18 °C. The lyophilized protein powder was immediately stored at −25 °C and used within 30 days of preparation. Protein denaturation during lyophilizing and cold storage was effectively suppressed by the addition of 0.3 M glucose (Saeki, 1997). In order to react protein with glucose through the Maillard reaction, the lyophilized protein powder was incubated at 40 °C and 65% relative humidity for 0−48 h. A humidity cabinet (model PR-1G, Tabai Espec Corp., Tokyo, Japan) was used to control the temperature and the relative humidity. In this study, myofibrillar proteins with 0.3 M sorbitol were also lyophilized and incubated under the same conditions as a model of unglycosylated protein.

Determination of Fructosamine and Available Lysine. Assays of fructosamine and available lysine were carried out

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Table 1. Available Lysine and Fructosamine Content of Glycosylated Myofibrillar Proteins[a]

reaction time (h)	available lysine (μmol/g)	fructosamine (μmol/g)
0	666	0
6	607	61
12	555	121
24	478	174
48	410	152

[a] Values given are averages of three determinations. Values in each sample are significantly ($p < 0.05$) different from one another.

for evaluating the protein glycosylation. The fructosamine was assayed by the method of Johnson et al. (1982), which is a simple spectrophotometric assay based on the property of fructosamine to act as a reductant in alkaline solution. Nitroblue tetrazolium was used as a substance, and glycosylated human serum was used as a standard for determining the fructosamine content. Available lysine content was determined by the spectrophotometric analysis using *o*-phthalaldehyde and *N*-acetyl-L-cysteine (Hernandez and Alvarez-Coque, 1992). Before the analysis of available lysine, the protein was precipitated with 7.5% trichloroacetic acid (at final concentration) to remove Tris buffer and redissolved in 50 mM phosphate buffer (pH 9.5) containing 2% sodium dodecyl sulfate (SDS) at room temperature. The assays were performed within 48 h after the protein glycosylation.

Solubility of Glycosylated Myofibrillar Proteins. After incubation, the protein powders were immediately mixed with 0.01−0.5 M NaCl containing 40 mM Tris-HCl (pH 7.5) at 2.0−2.5 mg/mL of the final protein concentration with a Potter-Elvehjem homogenizer at 120 rpm for 1 min and dialyzed in the same NaCl solution at 4 °C for 16 h. Unreacted glucose and sorbitol were removed at this step. A 10 mL amount of the dialyzate was placed in a 15 mL tube and centrifuged at 15000*g* for 30 min at 4 °C. The total solubility of myofibrillar proteins was expressed as percent of protein concentration in the supernatant with respect to that of the total protein solution before centrifugation.

Electrophoretic Analysis and Determination of Solubility of Myosin and Actin. Relative amounts of myosin and actin dissolved in 0.1 M NaCl (pH 7.5) were examined by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and quantified by densitometry. SDS−PAGE was performed in a 7.5% slab gel (9 cm × 8 cm) according to the method of Laemmli (1970). The sample was prepared as follows: 0.2 mL amounts of protein solutions were added to 0.8 mL of 2% SDS, 8 M urea, and 2% β-mercaptoethanol solution with 40 mM Tris-HCl (pH 8.0) and heated in boiling water for 2 min. Each 10 μL sample was loaded in each gel lane and 0.25% Coomassie Brilliant Blue R was used for protein staining. The image of each protein band stained on the gel was captured on a TIFF image file at 144 dpi and 256 gray scale by using a Macintosh model 7200/90 personal computer with an Epson GT-8000 scanner. NIH-Image, version 1.55 (computer software written by W. Rasband, U.S. National Institutes of Health, 1994), was used to determine the relative intensity of each protein band. The solubility of myosin and actin was expressed as percent of the relative intensity of the soluble fraction with respect to that of the protein solution before centrifugation. The solubility of myosin was determined by measuring the relative intensity of its heavy chain.

Statistical Analysis. All experiments were carried out with samples from three different lots of glycosylation. Values given in figures are the means of three determinations, and error bars indicate the standard deviation. Tukey's multiple range test (Steel and Torrie, 1986) was used to determine the significance of differences among samples shown in Table 1. Significance was determined at 95% level of probability.

RESULTS AND DISCUSSION

Reaction between Myofibrillar Proteins and Glucose. Table 1 shows the changes in available lysine and fructosamine contents in lyophilized myofibrillar proteins with glucose during reaction at 40 °C. The available lysine content decreased with reaction time, and fructosamine was produced simultaneously. The molar quantity of fructosamine produced was almost equal to that of the available lysine reduced during reaction for 6, 12, and 24 h. In addition, the available lysine content remained unchanged in the protein mixed with sorbitol, and no formation of fructosamine was observed under the same conditions (data not shown). These results indicate that glucose reacted with the lysine residue of myofibrillar proteins. However, in the reaction for 48 h, the formation of fructosamine was not observed while the decrease in available lysine continued. The decrease in fructosamine would indicate further progress of the Maillard reaction. Therefore, it is apparent that the Maillard reaction between the myofibrillar proteins and glucose was still in its early stages after 24 h of reaction.

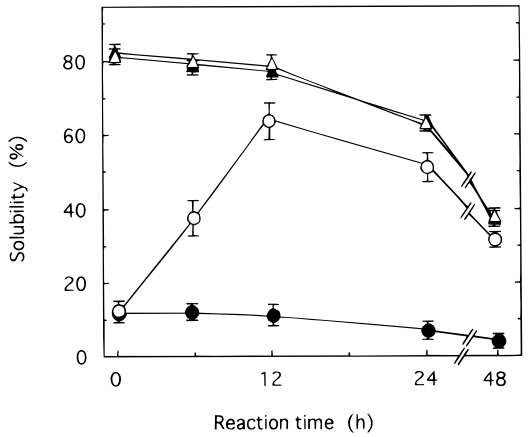

Figure 1. Changes in total solubility of myofibrillar proteins during glycosylation. Total solubility in 0.1 M (○) and 0.5 M (△) NaCl of myofibrillar proteins reacted with glucose. Total solubility in 0.1 M (●) and 0.5 M (▲) NaCl of myofibrillar proteins incubated with sorbitol.

Proteins Solubilized in Low Ionic Strength Medium with Glycosylation. Changes in the solubility of glycosylated proteins in 0.1 and 0.5 M NaCl are shown in Figure 1. Total solubility of myofibrillar proteins in 0.5 M NaCl was 81%, and it was almost unchanged during 12 h of Maillard reaction. Although the solubility then decreased with the progress of glycosylation, the same change was also observed in the total solubility of myofibrillar proteins with sorbitol incubated under the same conditions. Therefore, the decrease in the total solubility would be caused by heat denaturation of myofibrillar proteins, and glycosylation would have little effect on the total solubility in high ionic strength media when the Maillard reaction is still in its early stages. On the contrary, total solubility in 0.1 M NaCl increased markedly with glycosylation. It reached a maximum of 63%, when 17% of the available lysine was reacted with glucose, and the total solubility in 0.5 M NaCl was kept at a high level. Such an increase in the total solubility was not observed in the protein with sorbitol.

Figure 2 shows the SDS−PAGE patterns of the glycosylated protein and the soluble fraction in 0.1 M NaCl. Protein polymerization has often been observed when myofibrillar proteins reacted with glucose (Kim et al., 1984; Tanaka et al., 1994). However, the composition of myofibrillar proteins was almost unchanged by glycosylation, although tropomyosin disappeared with the progress of glycosylation. Apparently, the

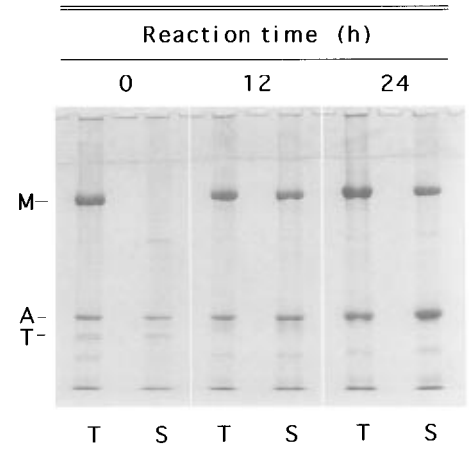

Figure 2. SDS−PAGE patterns of glycosylated myofibrillar proteins. Lane T: Myofibrillar proteins. Lane S: Soluble fraction in 0.1 M NaCl. M, Myosin heavy chain; A, actin; T, tropomyosin.

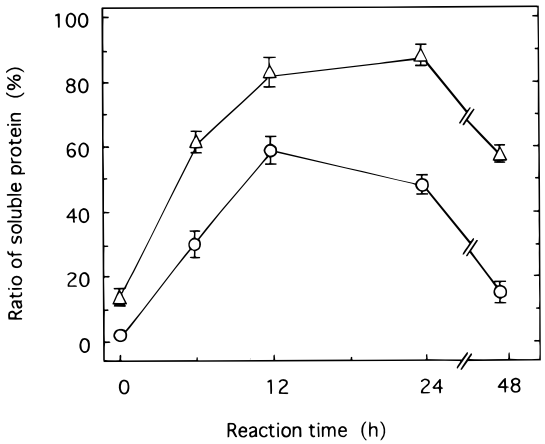

Figure 3. Protein components solubilized in low ionic strength medium. (○) Myosin; (△) actin.

disappearance of tropomyosin was not responsible for the change in solubility of myofibrillar protein because tropomyosin was only about 5% of the total protein (data not shown). Before glycosylation, small amounts of actin and tropomyosin were solubilized in 0.1 M NaCl. On the contrary, in glycosylated proteins, large amounts of myosin and actin were solubilized in 0.1 M NaCl. As described in the introduction, proteolysis of myofibrillar proteins often causes an increase in the myofibrillar proteins solubility in water (Lin and Park, 1996). However, no protein degradation was observed in the glycosylated proteins. Therefore, it is apparent that the marked increase in the total solubility in 0.1 M NaCl was due to the protein glycosylation.

As shown in Figure 3, the solubility of myosin and actin increased with glycosylation: 61% of myosin and 82% of actin were solubilized when myofibrillar proteins were glycosylated for 12 h. However, a marked decrease in the amount of solubilized myosin occurred with further progress of glycosylation. As described in Figure 1, glycosylation would have little effect on the solubility in high ionic strength media when the Maillard reaction is still in its early stages. These results indicate that the suppression of the thermal denaturation of myosin during the reaction with glucose would be important to maintain the high solubility in low ionic strength media.

Solubility of Glycosylated Proteins As Affected by NaCl Concentration. Figure 4 shows the effect of NaCl concentration on the total solubility of glycosylated

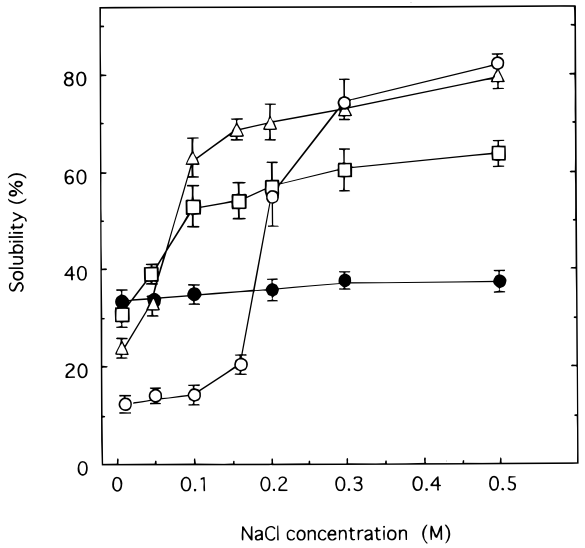

Figure 4. NaCl concentration dependence of total solubility of glycosylated myofibrillar proteins. Glycosylation time: 0 h (○), 12 h (△), 24 h (□), and 48 h (●).

myofibrillar proteins. The total solubility of unglycosylated myofibrillar proteins in 0.01−0.1 M NaCl solutions was less than 14% and a marked increase in the total solubility was observed in the range 0.16−0.3 M NaCl. On the other hand, the total solubility of glycosylated proteins increased remarkably in the range 0.01−0.1 M NaCl. Furthermore, the effect of NaCl concentration on the total solubility decreased with the progress of glycosylation even at high ionic strength. Such an effect of glycosylation on the protein solubility in NaCl solutions was also observed in KCl solution. For example, the total solubility in 0.16 M KCl also reached 71% after 12 h of reaction (result not shown). Therefore, it is apparent that the total solubility of myofibrillar proteins in a low ionic strength medium can be improved by protein glycosylation.

In conclusion, water-soluble myofibrillar protein can be prepared by glycosylation with the Maillard reaction. This information may contribute better utilization of fish myofibrillar proteins.

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