Preparation of Neoglycoprotein from Carp Myofibrillar Protein by Maillard Reaction with Glucose: Biochemical Properties and Emulsifying Properties

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Neoglycoprotein was prepared from carp myofibrillar protein by using the Maillard reaction with glucose, and its solubility in a high ionic strength medium, Ca-ATPase activity, and emulsifying properties were investigated. For reacting with glucose, the lyophilized protein mixed with glucose (the weight rate: 1:9) was incubated at 40 °C (for 24 h), 50 °C (for 6 h), or 60 °C (for 3 h) and 65% relative humidity. Fructosamine was produced during incubation, and available lysine content decreased simultaneously at all temperatures. In these conditions, the Maillard reaction occurring between a lysine residue and glucose was in the early stage. The glycosylated protein has high solubility in 0.5 M NaCl, and its emulsifying properties were superior to unglycosylated protein, although Ca-ATPase activity was completely lost. The regulation of the Maillard reaction in the early stage would be important for improvement of functional properties of fish myofibrillar protein.

Keywords: *Fish; myofibrillar protein; myosin; neoglycoprotein; glycosylation; Maillard reaction*

INTRODUCTION

Fish myofibrillar protein has excellent functional properties as foodstuffs, e.g., emulsifying properties, gel-forming ability, and water-holding capacity. However, the protein is thermally less stable than that of other vertebrates (Yamashita et al., 1978; Hashimoto et al., 1982). The functional properties are related to the solubilization of the protein in salt solutions, and they are lowered easily when protein denaturation occurs and the solubility in high ionic strength media of the protein is impaired (Kawashima et al., 1973; Numakura et al., 1989; Stefansson and Hultin, 1994). Therefore, the prevention of protein denaturation is very important when using fish muscle is used as foodstuffs.

Glycosylation of protein is an effective method for improving the functional properties of proteins. Synthetic glycoprotein is called neoglycoprotein, and many kinds of techniques are available for its preparation (Christopher et al., 1980). The Maillard reaction has often been used for glycosylation of proteins as a safe and mild modification method. Neoglycoproteins from ovalbumin (Kato et al., 1981), trypsin (Kato et al., 1993b), β-lactogroblin (Waniska and Kinsella, 1984), and borine serum albumin (Morales et al., 1976) were prepared by the Maillard reaction with glucose. The glycosylation of myofibrillar protein has also been studied (Yudkin et al., 1988; Brown et al., 1990; Syrovy and Hodny, 1992, 1993) to investigate the metabolic change with aging or diabetes. However, there has been no attempt to prepare neoglycoprotein from myofibrillar protein for improving its functional properties as a food material.

The object of this study was to prepare neoglycoprotein from fish myofibrillar protein by using the Maillard reaction with glucose for improving its functional properties. In this study, the improvement of emulsifying properties was investigated. The solubility in high ionic strength medium and the Ca-ATPase activity, which is measured for evaluating the quality of fish meat, were also studied.

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MATERIALS AND METHODS

Materials. A cultured live carp (*Cyprinus carpio*) was obtained at a local fish market. Adenosine 5′-triphosphate (ATP) was purchased from Pharmacia Biotech (Uppsala, Sweden). 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. Ordinary muscle from a carp was ground with a mean chopper and was suspended in three volume 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 impurities, the homogenate was centrifuged at 8000*g* for 10 min for collecting 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 Protein. Glucose at a final concentration of 0.3 M was added to the myofibrils. The protein concentration of the mixture was adjusted to 6.0 mg/mL. The mixture was 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.

In order to react protein with glucose through the Maillard reaction, the lyophilized protein powder was incubated at 40 °C for 0−24 h, 50 °C for 0−6 h, or 60 °C for 0−3 h and 65% relative humidity. An incubator/humidity cabinet (model LHU-112, Tabai Espec Corp., Tokyo, Japan) was used for controlling the temperature and relative humidity. After reacting with glucose, protein powder was immediately dis-

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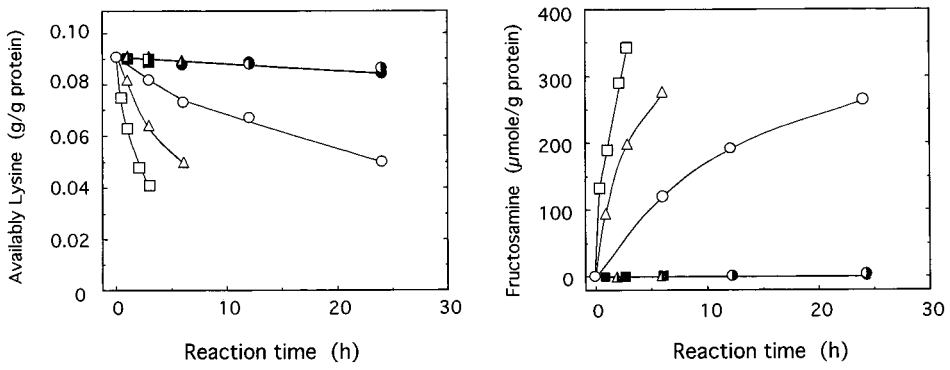

Figure 1. Decrease in available lysine and fructosamine formation in carp myofibrillar protein during reaction with glucose at different temperatures. Lyophilized myofibrillar proteins with glucose (○, △, □), with sorbitol (◑, ▲, ◨), and without sugar (●, ▲, ■) were incubated at 40 (○, ◑, ●), 50 (△, ▲, tus), or 60 °C (□, ◨, ■) and 65% relative humidity.

solved in 0.5 M NaCl−40mM Tris-HCl (pH 7.5) with a Potter-Elvehjem homogenizer, and unreacted glucose with protein was removed by dialysis against the same buffer at 4 °C. The dialyzed myofibrillar protein was used for subsequent experiments and analysis.

In this study, myofibrillar protein alone (without sugar) and myofibrillar protein with 0.3 M sorbitol were also lyophilized and incubated at the same condition as the protein with glucose.

Evaluation of Glycosylation. Fructosamine and available lysine assay were carried out for evaluating the protein glycosylation. The proteins dissolved in 0.5 M NaCl and 40 mM Tris-HCl (pH 7.5) was analyzed. The fructosamine assay was used by the method of Johnson et al. (1982). 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 precipiated with 7.5% (at final concentration) trichloroacetic acid 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.

Electrophoretic Analysis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed in a 5% and 7.5% slab gel according to the method of Laemmli (1970). Protein components were strained with Coomassie Brilliant Blue R.

Assessment of Solubility in High Ionic Strength Medium and Ca-ATPase Activity. Myofibrillar protein dialyzed against 0.5 M NaCl and 40 mM Tris-HCl (pH 7.5) for 16 h at 4 °C was centrifuged at 15 000*g* for 30 min. Solubility was expressed as percent of protein concentration in the supernatant with respect to that of the total protein solution before centrifugation. The Ca-ATPase assay was performed at 25 °C in a mixture containing 0.5 M NaCl, 5 mM $CaCl_2$, 1 mM ATP, 25 mM Tris-maleate (pH 7.0), and 0.2−0.3 mg/mL of protein. The reaction was stopped by adding $HClO_4$ to a final concentration of 5%. The inorganic phosphate liberated was measured by the method of Gomori (Gomori, 1942). The myofibrillar Ca-ATPase specific activity was expressed as μmol of P_i liberation min^{-1} (mg of protein)$^{-1}$. There were no differences in the Ca-ATPase activity between total protein solution before centrifugation and the supernatant.

Analysis of Emulsifying Properties. The protein dissolved in 0.5 M NaCl, 40 mM Tris-HCl (pH 7.5) was examined. Emulsifying properties of the salt-soluble fraction were determined according to the method of Pearce and Kinsella (1978). 2.1 mL of protein solution (1.0 mg/mL) was mixed with 0.7 mL of corn oil in a test tube (diameter: 10.5 mm), and an oil-in-water emulsion was prepared using a homogenizer (Ultra-turrax T 25/N-8G, IKA-LABORTECHNIK, Staufen, Germany) operating at 13 500 rpm for 1 min in ice water. After standing for 0, 5, 10, 20, 30, and 60 min in an ice water bath, 0.15 mL aliquots of the emulsion were taken from the bottom of the test tube and diluted with 2.85 mL of 0.1% SDS incubated at 25 °C. The turbidity of each diluted emulsion was immediately measured at 500 nm with a 1-cm pathlength cuvette for evaluating the emulsifying stability. Emulsifying stability was expressed by measuring the time required for the turbidity to be reduced to one-half its initial value. Pearce and Kinsella reported that creaming occurs by removing oil globules from the bulk of the emulsion (Pearce and Kinsella, 1978). Creaming which occurred in the emulsion was then observed with the naked eye.

Statistic Analysis. To evaluate the improvement of emulsifying properties, Tukey's multiple range test (Steel and Torrie, 1986) was used to determine the significance of differences among samples.

RESULTS AND DISCUSSION

Production of Fructosamine Occurring with Decrease in Available Lysine and SDS−PAGE Pattern. As shown in Figure 1, fructosamine was produced in the lyophilized protein with glucose during incubation and the available lysine content decreased simultaneously at all temperatures. The molar quantity of the fructosamine studied was almost equal to that of the available lysine reduced. The increase in fructosamine and the decrease in available lysine occurred rapidly with an increase in the reaction temperature. However, when the lyophilized protein without sugar or with sorbitol was incubated under the same condition, the available lysine content remained unchanged and the production of fructosamine was not observed. These results indicate that the neoglycoprotein was prepared from myofibrillar protein through the Maillard reaction.

SDS−PAGE patterns of myofibrillar protein glycosylated with glucose at 60 °C are shown in Figure 2. In 5% acrylamide gel, a slight decrease in electrophoretic mobility of myosin heavy chain was observed, and disappearance of tropomyosin was also noticed with a progress of glycosylation. The same changes in SDS−PAGE patterns of myofibrillar protein were observed with the progress of protein glycosylation at 40 and 50 °C. It was previously reported that a polymerization and brown discoloration of myosin heavy chains occurred in bovine myofibrils (Kim et al., 1984) and squid meat (Tanaka et al., 1994) with a progress of the Maillard reaction with glucose. However, no polymerization occurred under such conditions of protein glycosylation and no brown discoloration was observed either (results not shown). Further, the fructosamine, which is the amadori rearrangement product in the early stage of the amino−carbonyl reaction, was monotonically increased as shown in Figure 1. These results indicate that the Maillard reaction between lysine

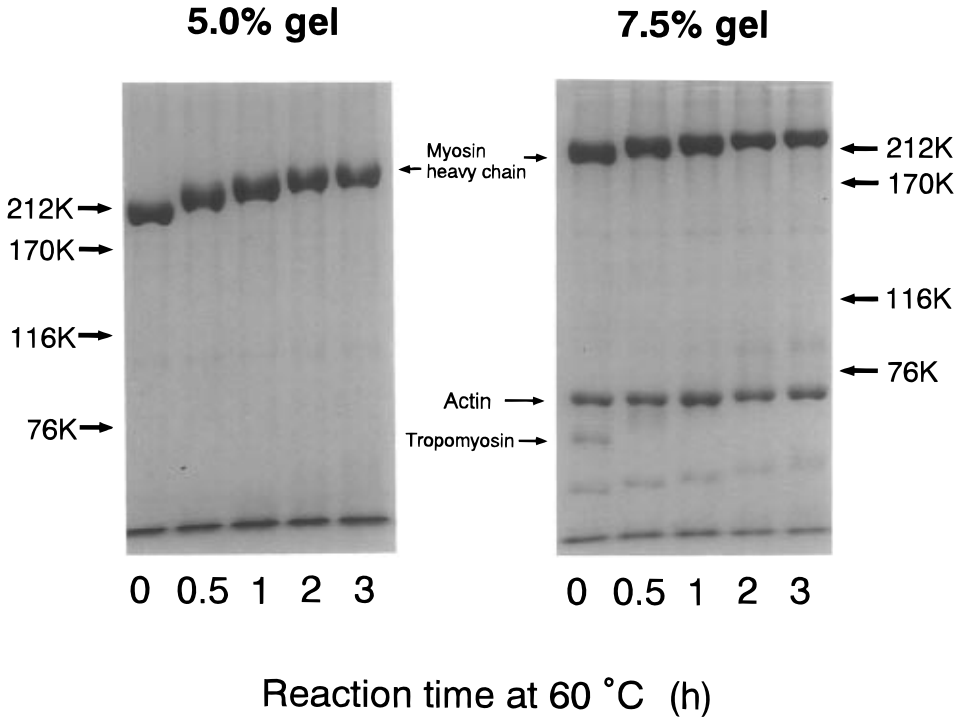

Figure 2. SDS−PAGE patterns of glycosylated myofibrillar protein.

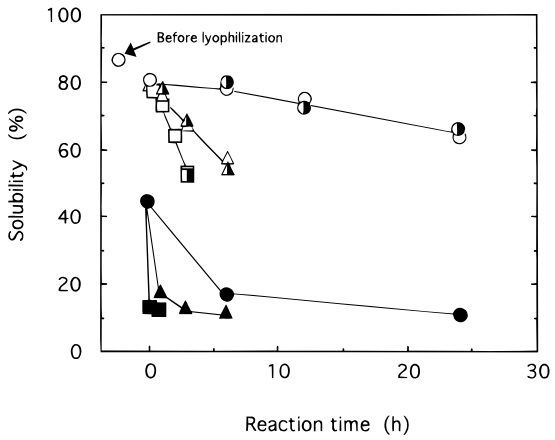

Figure 3. Change in solubility of myofibrillar protein during glycosylation at different temperatures. Symbols are as in Figure 1.

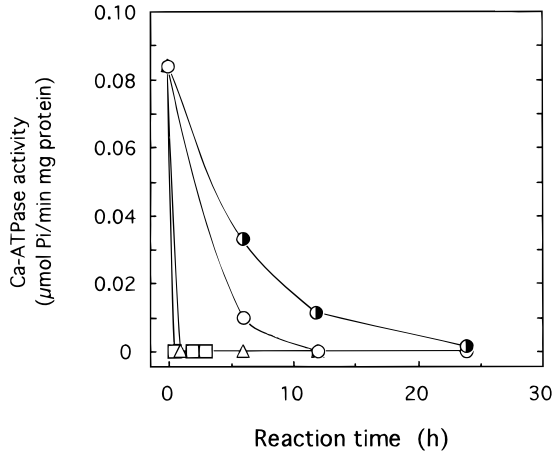

Figure 4. Change in Ca-ATPase activity of myofibrillar protein during glycosylation at different temperatures. Symbols are as in Figure 1.

residue of myofibrillar protein and glucose was in the early stage.

Change in Biochemical Properties of Myofibrillar Protein with Glycosylation. Figure 3 shows the change in the solubility of the protein with glucose, with sorbitol, and without sugar during incubation at 40−60 °C and 65% relative humidity. Before lyophilization, the solubility of myofibrillar protein was 86%. After lyophilization, the solubility of the protein without sugar decreased up to 44% and a further lowering occurred during incubation. On the contrary, the solubility of the lyophilized protein with glucose or with sorbitol was 81%, and only a slight decrease was observed when incubated at 40 °C for 24 h. The solubility was also maintained more than 50% in reaction at 50 °C for 6 h and 60 °C for 3 h, although it diminished more rapidly. Despite about 50% of lysine was converted into fructosamine in the glycosylated protein, its solubility was almost the same as that of the protein with sorbitol. This result indicates that the glycosylation has little effect on the solubility of carp myofibrillar protein at the early stage of the Maillard reaction. Glucose would depress the protein denaturation during lyophilization and glycosylation the same as sorbitol.

As presented in Figure 4, Ca-ATPase activity of glycosylated myofibrillar protein rapidly decreased, although its solubility was kept at a high level as presented in Figure 3. It was lost within 1 h at 50 and 60 °C. Koseki et al. (1993) reported that a decrease in solubility of heat-treated myofibrillar protein occurred rapidly before inactivation of its Ca-ATPase, and they proposed that myosin solubility in a high ionic strength medium would be suitable as a sensitive indicator for thermal denaturation of myofibrillar protein. However, as shown in Figures 3 and 4, myofibrillar Ca-ATPase activity was completely lost before the decrease in its solubility. In skeletal muscle myosin, reactive lysine is located in the active site of ATPase activity (Miyanishi et al., 1979), and it was reported that the chemical modification of lysine residue of myosin head resulted in a marked decrease in the Ca-ATPase (Kubo et al.,

Table 1. Improvement of Emulsifying Properties of Myofibrillar Protein by Glycosylation

specimen		fructosamine content	decreasing rate of	emulsifying activity	emulsifying
sugar	treatment[a]	(μmol/g)	available lysine (%)	(OD at 500 nm)	stability[b] (min)
glucose	none	0	0	1.37 a	5> a
	40 °C/24 h	265	42.3	1.77 b	10.0 b
	50 °C/6 h	272	43.7	1.80 b	18.4 c
	60 °C/2 h	289	46.7	1.85 b	19.0 c
sorbitol	none	0	0	1.41 a	5> a
	40 °C/24 h	0	3.5	1.12 a	5> a
	50 °C/6 h	0	2.3	1.20 a	5> a
	60 °C/2 h	0	6.0	1.00 b	5> a

[a] Relative humidity, 65%. [b] Different letters signify a significant difference.

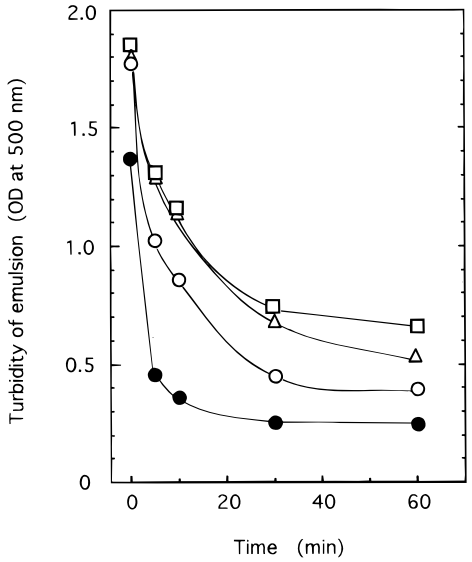

Figure 5. Comparison of emulsifying properties of glycosylated myofibrillar proteins. Myofibrillar proteins (●) were reacted with glucose at 40 °C for 24 h (○), 50 °C for 6 h (△), and 60 °C for 2 h (□).

1960). As shown in Figure 4, the loss of Ca-ATPase activity of the glycosylated protein occurred faster than that of the protein with sorbitol. Thus, the marked inactivation of the Ca-ATPase could be caused by the modification of reactive lysine located in the active site of ATPase activity.

Improvement of Emulsifying Properties by Glycosylation. Figure 5 shows the emulsifying properties of glycosylated myofibrillar protein. Compared with the glycosylated proteins, the turbidity of emulsion of the unglycosylated one rapidly decreased. In addition, a cream layer was formed clearly in the emulsion of unglycosylated myofibrillar protein within 8 min after emulsifying. In contrast, the turbidity decreased more slowly in the glycosylated proteins and such creaming did not occur. Therefore, these results indicate that the emulsifying activity of the glycosylated proteins was higher than that of unglycosylated one. As shown in Table 1, emulsifying stability also increased 2−4 times by the glycosylation. On the other hand, the lyophilized protein with sorbitol was incubated under the same condition (its solubility was almost the same as the glycosylated protein as shown in Figure 3). However, there was no improvement of emulsifying properties. It is therefore apparent that the improvement of the emulsifying properties was caused by the protein glycosylation.

Improvement of emulsifying properties by glycosylation of protein was reported in conjugates of egg white protein−galactomannan (Kato et al., 1993a), casein−dextran (Kato et al., 1992), ovalbumin−glucose 6-phosphate (Kato et al., 1995), and protamine−galactomannan (Matsudomi et al., 1994). In these cases, the Maillard reaction proceeded in the advanced stage because protein polymerization or browning were observed. On the contrary, in carp myofibrillar protein, the improvement of emulsifying properties was achieved through the early stage of the Maillard reaction. More progress of the Maillard reaction would result in loss of solubility as shown in Figures 1 and 3. Therefore the regulation of the Maillard reaction in the early stage is very important for improvement of functional properties of fish myofibrillar protein.

CONCLUSION

Neoglycoprotein was prepared from carp myofibrillar protein by using the Maillard reaction. The solubility in a high ionic strength medium was maintained at a high level although the Ca-ATPase was lost. The emulsifying properties of myofibrillar protein was improved by glycosylation through the early stage of the Maillard reaction.

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