Effect of Maillard Reaction with Glucose and Ribose on Solubility at Low Ionic Strength and Filament-Forming Ability of Fish Myosin

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It was previously reported that water-soluble fish meat can be prepared by the Maillard reaction with reducing monosaccharides. To clarify the molecular mechanism of the solubility improvement caused by the glycosylation, carp myosin and myosin rod region were reacted with glucose and ribose, and their solubilities and filament-forming abilities at low ionic strength were investigated. The solubility of myosin in 0.1 M NaCl increased with the glycosylation and reached the same level as in 0.5 M NaCl. Although the same solubility improvement was observed in glycosylated myosin rod and the α-helix content remained unchanged, the filament-forming ability was completely lost by the glycosylation. These results suggest that the solubility improvement of fish meat at low ionic strength is caused by the solubilization of myosin and the dissociation of myosin filaments.

Keywords: *Fish; myosin; myosin rod; glycosylation; Maillard reaction; filament formation; solubility; glucose; ribose*

INTRODUCTION

Fish meat is an abundant protein resource that is widely used in food products. Fish myofibrillar protein has excellent functional characteristics such as emulsifying properties, gel-forming ability, and water-holding capacity. Many kinds of seafood products, ready-to-fry fish portions, surimi-based products, and fish sausages and cakes take advantage of the functional properties of fish myofibrillar protein. However, fish myofibrillar protein is thermally and chemically less stable than that of other vertebrates (*1*, *2*), and its functional properties are lowered easily when protein denaturation occurs. Because the solubility in high ionic strength media of the protein is impaired with the progress of protein denaturation, the solubility of muscle proteins in salt solutions has been discussed from the point of view of the quality of fish meat as a food material (*3*−*5*).

It has been reported that protein glycosylation with reducing monosaccharides using the Maillard reaction is an effective method to improve the functional properties of food proteins (*6*−*8*). We have also reported in previous papers (*9*, *10*) that carp myofibrillar protein became solubilized in low ionic strength media when it reacted with glucose or ribose in the early stage of the Maillard reaction. Because no protein degradation occurred during the glycosylation, the water-soluble myofibrillar protein had excellent emulsifying properties (*11*).

It is known that the ionic strength dependence of the solubility of fish meat reflects the biochemical characteristics of myosin, which is the major protein in muscle. Therefore, some functional changes in myosin probably induce the solubility improvement of fish muscle by the glycosylation. Myosin can be split into two fragments, namely, the water-soluble head region (subfragment 1) and the water-insoluble rod region. Myosin molecules spontaneously assemble into insoluble filaments in solutions of physiological ionic strength and neutral pH, and the myosin rod region is the most important structural part of myosin filaments. In this study, the effect of glycosylation on the solubility of myosin and the filament-forming ability of the myosin rod region were investigated to clarify the molecular mechanism of the solubility improvement of fish meat.

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MATERIALS AND METHODS

Materials. Cultured live carps (*Cyprinus carpio*; 1.0−1.2 kg) were obtained at a local fish market. Sephacryl S-500 was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Fructosamine test calibration kit (for ketoamine assay) was purchased from Japan Roche Co. Ltd. (Tokyo, Japan). All other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Myosin and Myosin Rod. All preparations were carried out below 8 °C. Carp myofibrils were prepared from fresh dorsal muscle according to the method of Saeki et al. (*11*). Myosin was extracted from the myofibrils with 0.5 M KCl containing 2 mM ATP, 2 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.5) and purified by ammonium sulfate fractionation (*12*). Myosin rod was prepared from the myofibrils by limited chymotryptic digestion (*13*). The prepared myosin and myosin rod were suspended in 50 mM NaCl (without Tris buffer) with a Teflon potter homogenizer. Protein concentration was measured according to the biuret method (*14*) with bovine serum albumin (fraction V) as a standard.

Glycosylation of Myosin and Myosin Rod. Myosin and myosin rod were mixed with the glucose and ribose at final concentrations of 0.6 and 0.3 M, respectively. The protein concentration was adjusted to 3 mg/mL. The mixtures were placed into test tubes, frozen at −40 °C, and immediately lyophilized. The lyophilization was stopped when the sample temperature reached 14 °C. The lyophilized protein powders were immediately stored at −30 °C and used within 20 days of preparation. To react the proteins with the monosaccharides through the Maillard reaction, the lyophilized protein powders were incubated at 30−40 °C (myosin) or 35 °C (myosin rod) and 65% relative humidity. A humidity cabinet (model PR-

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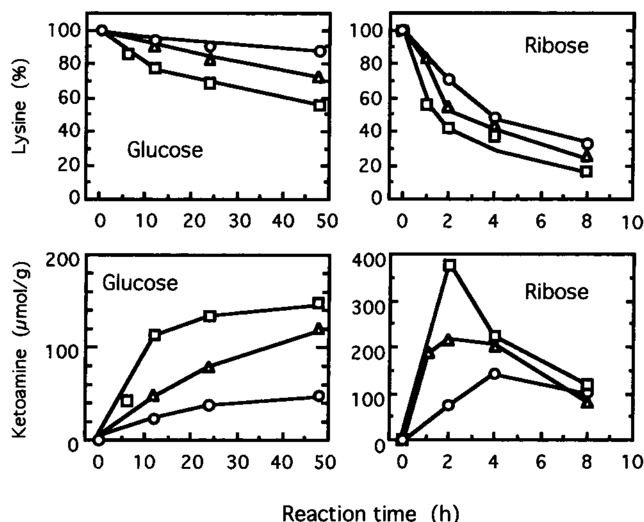

Figure 1. Changes in available lysine and ketoamine contents in myosin during reaction with glucose and ribose. Reaction temperatures: ○, 30 °C; △, 35 °C; □, 40 °C. The coefficient of variation in replicates is <6% ($n = 4$).

1G, Tabai Espec Corp., Tokyo, Japan) was used to control the temperature and relative humidity.

Determination of Available Lysine and Ketoamine Contents. Proteins were dissolved in 0.5 M NaCl containing 40 mM Tris-HCl (pH 7.5), and the available lysine and ketoamine contents were measured to evaluate the protein glycosylation by using the methods previously described (9). A fructosamine test calibration kit was used as a standard for determining the ketoamine content.

Solubility of Glycosylated Proteins. After incubation, the protein powders were immediately dissolved in 0.1 and 0.5 M NaCl solutions containing 40 mM Tris-HCl (pH 7.5) at 0.4–0.7 mg/mL of the final protein concentration with a homogenizer. Each homogenate was dialyzed against the same NaCl solution at 4 °C for 16 h and centrifuged at 15000*g* for 30 min at 4 °C. Protein solubility was expressed as percentage of protein concentration in the supernatant with respect to that of the total protein solution before centrifugation. Protein concentration was measured according to the microbiuret method (*15*). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*16*) was performed to investigate the protein subunit change occurred by glycosylation.

Estimation of α-Helix Content of Myosin Rod. Glycosylated myosin rod was dissolved in 0.1 or 0.5 M NaCl solution containing 5 mM 2-mercaptoethanol and 40 mM Tris-HCl (pH 7.5), and the circular dichroism spectrum was measured at 20 °C. α-Helical content was estimated by the equation (*17*)

$$\text{content (\%)} = -([q] + 3000)/39000 \times 100$$

where [q] is the molecular ellipticity at 222 nm.

Gel Permeation Chromatography of Glycosylated Myosin Rod. Glycosylated myosin rod dissolved in 0.1 or 0.5 M NaCl and 20 mM Tris-HCl (pH 7.5) was loaded onto a Sephacryl S-500 column (16 × 90 cm) equilibrated with the same buffer at a flow rate of 0.4 mL/min.

Each 2 mL of fraction was collected, and the protein concentration was determined according to the Lowry method (*18*).

RESULTS AND DISCUSSION

Solubility Changes in Myosin by Glycosylation. Figure 1 shows the progress of glycosylation in myosin. When the mixtures of myosin and monosaccharides were incubated at 30–40 °C, the available lysine in myosin decreased and ketoamine was simultaneously produced at all temperatures. In the early stage of the Maillard reaction, monosaccharides bound to proteins by aldimine linkage underwent an Amadori rearrangement to the ketoamines (*19*). Thus, the results of Figure 1 indicate that lysine residues in myosin reacted with glucose and ribose through the Maillard reaction. Ribose was rapidly reacted with the available lysine of myosin rather than glucose at all temperatures, and the ketoamine decrease was observed in the prolonged incubation of the myosin–ribose system. Ribose is more reactive than glucose in forming condensation products with amino groups of proteins (*20*). Therefore, the decrease in ketoamine in the myosin–ribose system would be caused by the progress of the Maillard reaction.

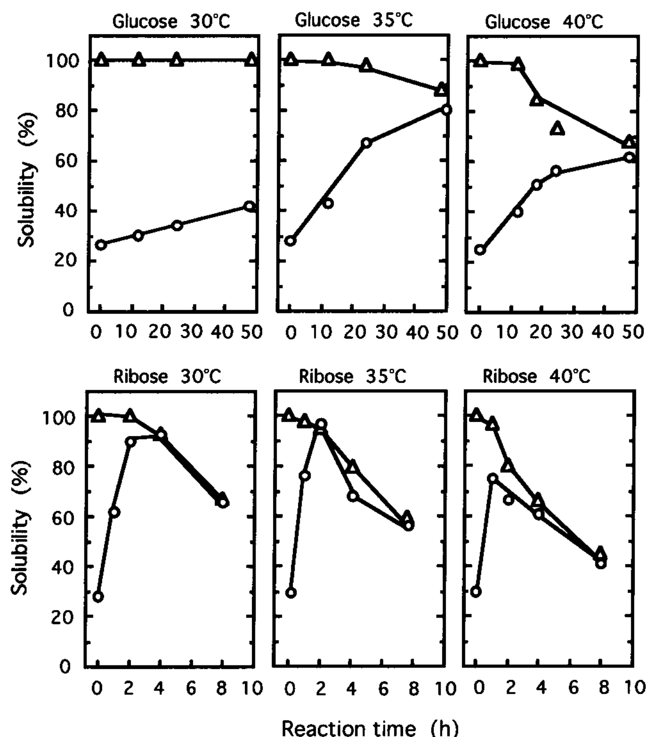

Figure 2. Effect of glycosylation on solubility of myosin: solubility in 0.1 M (○) and 0.5 M (△) NaCl. The coefficient of variation in replicates is <4% ($n = 3$).

Figure 2 shows the solubility change of myosin by the reaction with the monosaccharides. The solubility of myosin in 0.1 M NaCl increased greatly with the progress of the glycosylations, except for the reaction with glucose at 30 °C. In the reaction with glucose, a marked enhancement of the solubility was observed at 35 °C. When 28% of the available lysine was reacted with glucose at 35 °C for 48 h, the solubility in 0.1 M NaCl reached 96%, which was equal to the solubility in 0.5 M NaCl. Furthermore, the solubility of myosin rapidly increased in the reaction with ribose at all temperatures. When reacted at 35 °C for 2 h, the available lysine loss was 28%, and the solubility in 0.1 M NaCl reached 91%. No protein degradation occurred in the glycosylated myosins by SDS-PAGE (data not shown). Thus, it is apparent that the increase in myosin solubility in a low ionic strength medium was caused by introducing monosaccharides into the myosin molecules.

When myosin was reacted with ribose, the improved solubility in 0.1 M NaCl decreased with the loss of 0.5 M NaCl. It would be caused by the production of insoluble protein–sugar complex with the progress of

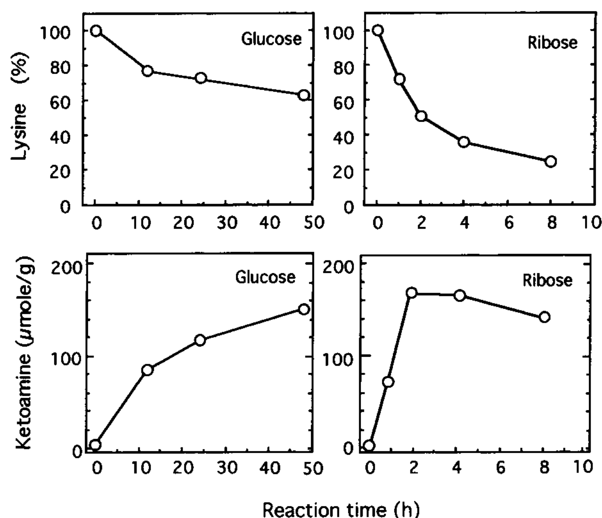

Figure 3. Changes in available lysine and ketoamine contents in myosin rod during reaction with glucose and ribose at 35 °C. The coefficient of variation in replicates is <4% ($n = 4$).

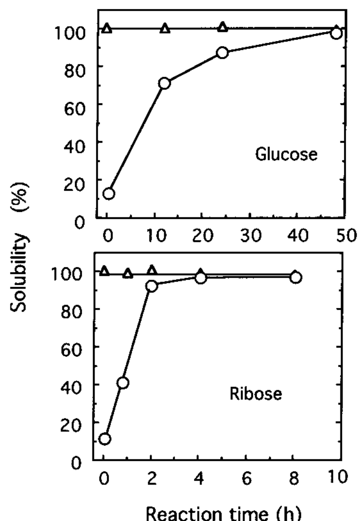

Figure 4. Effect of glycosylation on solubility of myosin rod: solubility in 0.1 M (○) and 0.5 M (△) NaCl. The coefficient of variation in replicates is <5% ($n = 3$).

the Maillard reaction. This result suggests that the regulation of the Maillard reaction in the early stage is important in the improvment of the functional properties of fish myofibrillar protein.

Changes in Solubility of Myosin Rod by Glycosylation. When myosin rod was reacted with glucose and ribose at 35 °C, a decrease in the available lysine and an increase in ketoamines were observed, as shown in Figure 3. When the effect of glycosylation on the solubility of myosin rod in 0.1 and 0.5 M NaCl was investigated as shown in Figure 4, a marked enhancement of the solubility in 0.1 M NaCl was observed in myosin rod. The time course of the solubility increase was similar to the result of the glycosylated myosin shown in Figure 2. When 21% of the available lysine in myosin rod was reacted with glucose at 35 °C for 24 h, the improved solubility in 0.1 M NaCl reached 91%. In the reaction with ribose, the same effect by glycosylation was obtained at 35 °C for 2 h. It is known that although the head region of myosin is water-soluble, myosin is a water-insoluble protein. Therefore, the result of Figure 4 clearly indicates that the increase in the solubility of myosin at low ionic strength was caused by the solubility improvement of the rod region.

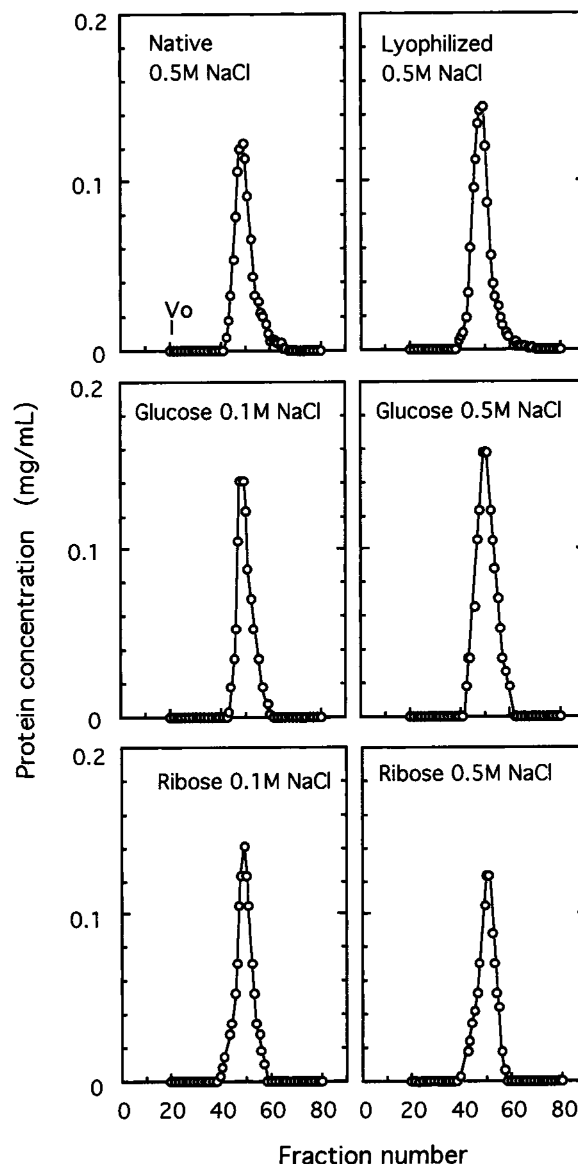

Figure 5. Elution profiles of myosin rod. Myosin rod was reacted with glucose and ribose at 35 °C for 24 and 4 h, respectively. As a control, native myosin rod and lyophilized myosin rod with glucose (no reaction) were examined. V_0: void volume decided by actomyosin.

As shown in Figure 4, no solubility decrease was observed in the myosin rod during the Maillard reaction with monosaccharides. Therefore, it is apparent that the loss of myosin solubility was caused by a decrease in solubility of the head region. It is known that the progress of the Maillard reaction occurring between proteins and reducing sugars results in polymerization of proteins and subsequent loss of their solubility (*21, 22*). However, no protein polymerization was observed in glycosylated myosin shown in Figure 2 (by SDS-PAGE analysis, data not shown). The structure of the head region of fish myosin is less stable than that of the rod region, and it aggregates easily by heating or by chemical treatments (*23, 24*). Thus, the structural change in the head region would be accelerated by the glycosylation.

Loss of Filament-Forming Ability of Myosin Rod by Glycosylation. Myosin rods reacted with glucose and ribose were subjected to gel permeation chroma-

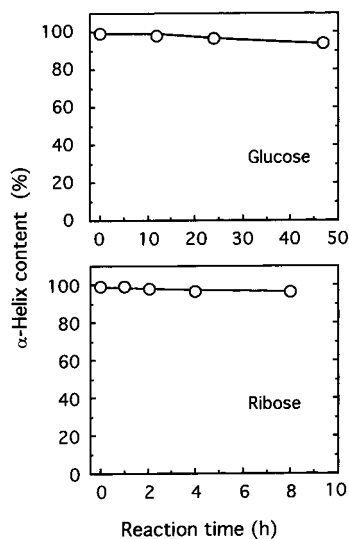

Figure 6. α-Helix content of glycosylated myosin rod. The coefficient of variation in replicates is <3% ($n = 2$).

tography to investigate the soluble state in a low ionic strength medium (Figure 5). Both glycosylated myosin rods were eluted as a single peak in 0.5 M NaCl, and their elution pattern coincided with that of native and lyophilized myosin rods. The same elution pattern was also obtained in the glycosylated myosin rods solubilized in 0.1 M NaCl. Native myosin rod is solubilized in high ionic strength media (>0.3) as a monomer, whereas it aggregates and assembles into insoluble filaments in low ionic strength media. Therefore, Figure 5 indicates that the glycosylated myosin rod lost its filament-forming ability and existed as a monomer regardless of the ionic strength. In addition, no change was observed in the α-helix content in the myosin rods when they were reacted with the monosaccharides, as shown in Figure 6. It is known that the structure of myosin rod is a two-strand α-helical coiled coil and that the lysine residues in myosin rod are located on the periphery of the coiled coil (*25*). Because solubilization of myosin at low ionic strength was achieved without substantial conformational change in the rod region, monosaccharides bound to lysine residues would be located on the surface of the rod molecule and inhibit the filament formation of myosin rod.

In this study, we concluded that myosin was solubilized at low ionic strength by introducing monosaccharides into the rod region. Furthermore, it was found that the filament-forming ability of myosin was impaired by the glycosylation. These results suggest that the improved solubility of fish meat at low ionic strength by glycosylation is caused by the solubilization of myosin and the dissociation of myosin filaments in myofibrils.

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