Preparation of Neoglycoprotein from Carp Myofibrillar Protein and Alginate Oligosaccharide: Improved Solubility in Low Ionic Strength Medium

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Alginate oligosaccharide (AO) was conjugated with carp myofibrillar protein (Mf) by using the controlled Maillard reaction, and the change in the solubility of Mf in low ionic strength media as affected by the glycosylation was investigated. AO was prepared by degrading sodium alginate using alginate lyase, which was purified from the culture supernatant of *Pseudoalteromonas elyakovii*. When a lyophilized Mf and AO mixture was incubated at 40 °C and 65% relative humidity, the conjugation of AO was confirmed at myosin heavy chain, actin, and tropomyosin. When >30 μg/mg of AO was conjugated to Mf, the protein solubility in a low ionic strength medium was greatly improved without significant loss of available lysine. These results indicate that the conjugation with AO is a superior manner for improving the water solubility of Mf in view of its nutritional aspect.

Keywords: *Fish; myofibrillar proteins; neoglycoprotein; glycosylation; Maillard reaction; alginate oligosaccharide; solubility; Ca-ATPase activity*

INTRODUCTION

Protein glycosylation is an effective method for improving the functional properties of food proteins. Techniques using various kinds of cross-linkers and modified reagents have been developed to prepare synthetic glycoproteins (neoglycoproteins). Recently, a simple synthetic method using the Maillard reaction has been proposed, in which ϵ-amino groups in lyophilized protein react with the reducing end carbonyl group in carbohydrates under heat treatment at relatively low temperatures. Neoglycoproteins from ovalbumin, β-lactoglobulin, bovine serum albumin, lysozyme, gluten, protamin, and phosvitin have been prepared by conjugation with polysaccharides using the Maillard reaction, and these neoglycoproteins are superior to native proteins in functional properties such as emulsifying property, thermal stability, antimicrobial action, and antioxidant effect (Kato et al., 1990, 1991; Matsudomi et al., 1994; Shu et al., 1998; Nakamura et al., 1998; Babiker et al., 1998).

Fish myofibrillar protein has excellent functional properties as foodstuffs, for example, emulsifying properties, gel-forming ability, and water-holding capacity. Attempts have been made to improve such characteristics by protein glycosylation. For instance, a water-soluble myofibrillar protein with an enhanced emulsifying property was prepared by modification of lysine residues with glucose (Saeki, 1997; Saeki and Inoue, 1997). Furthermore, Fujiwara et al. (1998) reported that the emulsifying property and thermal stability of carp myofibrillar protein were improved by conjugation with dextran. These findings indicate that protein glycosylation is useful for improving the functional properties of fish myofibrillar proteins.

The final objective of this study is to propose a new manner of marine bioresource utilization by preparing neoglycoproteins with new functional properties using marine bioproducts. In this work, carp myofibrillar protein was conjugated with oligosaccharide of sodium alginate from brown algae prepared with marine bacterial alginate lyase, and the changes in the solubility in low ionic strength media and some biochemical properties of the protein as affected by the glycosylation were investigated. The results will be discussed in comparison to the glycosylation with glucose and dextran.

MATERIALS AND METHODS

Materials. A cultured live carp (*Cyprinus carpio*) was obtained at a local fish market. Sodium alginate from brown algae was purchased from Kanto Chemicals Co., Inc. (Tokyo, Japan), D-mannuronic acid and Coomassie Brilliant Blue R were purchased from Sigma (St. Louis, MO), and adenosine 5′-triphosphate (ATP) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Myofibrillar Protein. Carp myofibrillar protein (Mf) was prepared from ordinary muscle according to the method previously described (Saeki, 1997) and suspended in 50 mM NaCl.

Preparation of Alginate Oligosaccharide. Alginate oligosaccharide (AO) was prepared by degrading sodium alginate using alginate lyase, which was purified from the culture supernatant of *Pseudoalteromonas elyakovii* (IAM 14594) (Sawabe et al., 1992). $MgCl_2$ (50 mM) and 1000 units/L of the alginate lyase were added to a 3% sodium alginate solution. The mixture was gently stirred and incubated at 30 °C for 96 h. The AO thus obtained was filtered through a paper filter, concentrated with a rotary evaporator, and then mixed with 80% ethyl alcohol. The AO, which had been collected as a precipitate by centrifugation, was resuspended in 80% ethyl alcohol five times to remove $MgCl_2$. After being redissolved in distilled water, AO was ultrafiltrated using a polymer mem-

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10.1021/jf990081m CCC: $19.00
Published on Web 12/16/1999

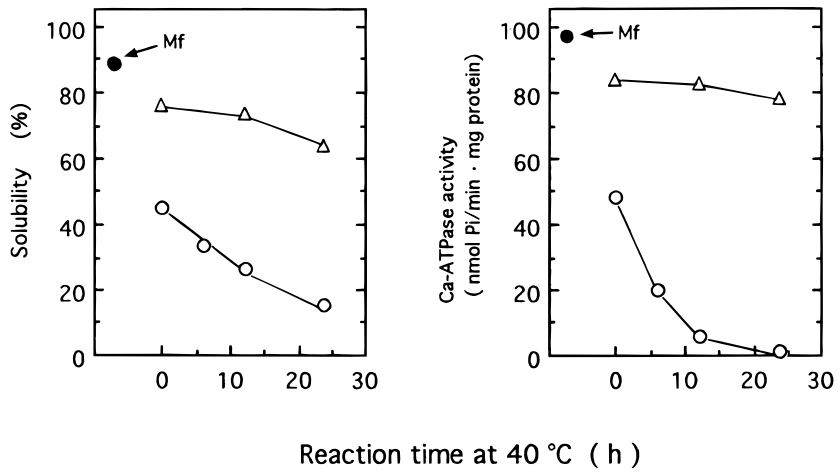

Figure 1. Changes in solubility in 0.5 M NaCl and Ca-ATPase activity of carp Mf protein during lyophilization and incubation at 40 °C. Mf−AO mixture containing 0 M (○) and 0.6 M sorbitol (△) was lyophilized and further incubated at 40 °C. (●) indicates the Mf data before lyophilization.

Table 1. Composition of AO

DP	%	DP	%
4 and 5	12.6	7	51.0
6	35.2	>8	1.2

brane (MW cut-off limit = 50000) to remove undegradated alginate and was then lyophilized in a freeze-dryer (FDU-506, Tokyo Rika Co., Ltd., Tokyo, Japan).

The degree of polymerization (DP) of AO was determined by gel filtration analysis using a Cellulofine GCL-25m column (∅ 1.8 × 70 cm, Seikagaku Kogyo Co., Ltd.) eluted with 0.1 M Na_2SO_4 at 20 mL/h. Molar concentration (*A*) of AO in each fraction was measured by the phenol−sulfuric acid reaction (Dubois et al., 1956) using D-mannuronic acid lactate as a standard. The concentration of the reducing end group in AO of the same fraction (*B*) was determined according to the Somogyi method (Somogyi, 1952), and DP was calculated as *A*/*B*. The composition of AO is listed in Table 1; the overall DP (the mean value of total fractions) was 6.1.

Neoglycoprotein Synthetic System between Mf and AO. Carp Mf suspended in 50 mM NaCl containing 0 or 0.6 M sorbitol was mixed with AO at different weight ratios as described under Results and Discussion. The final concentration of protein was adjusted to 6.0 mg/mL, and AO was also adjusted to 2.4, 6.0, 24.0, or 54.0 mg/mL (Mf/AO = 1:0.4, 1:1, 1:4, and 1:9). The protein concentration was determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard. Two milliliters of the mixtures placed in test tubes (diameter = 11 mm) was frozen at −40 °C and immediately lyophilized in the freeze-dryer.

The lyophilization was stopped when the sample temperature reached 14 °C. The lyophilized Mf mixed with AO thus obtained (Mf−AO mixture) was immediately stored at −25 °C and used within 30 days of preparation.

Glycosylation of Mf was performed in a controlled dry state. Mf−AO mixture was incubated at 40 °C and 65% relative humidity in a humidity cabinet (model PR-1G, Tabai Espec Corp., Tokyo, Japan). After reaction, Mf conjugated with AO (Mf−AO conjugate) was dissolved in different concentrations of NaCl by the following process.

Solubility. Mf−AO was suspended in 0.05−0.5 M NaCl containing 40 mM Tris-HCl (pH 7.5) at 1.0 mg/mL of the final protein concentration with a homogenizer (Ultra-turrax T 25/ N-8G, IKA- Labortechnik, Staufen, Germany) operating at 13500 rpm for 60 s and immediately centrifuged at 15000*g* for 30 min at 4 °C. The supernatant and the total protein solution were mixed with an equal volume of 2.0 M NaOH, and their protein concentrations were determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

In this work, total solubility of Mf was investigated because Mf consists of various kinds of proteins, such as myosin, actin, and tropomyosin. The total solubility was expressed as percent of protein concentration in the supernatant with respect to that of the total protein solution before centrifugation. In the preliminary experiment, we confirmed that unreacted AO has no effect on the total solubility of Mf−AO conjugate.

Assays of Ca-ATPase Activity, Amount of AO Bound to Mf, and Available Lysine Content. Sixty percent of saturated ammonium sulfate at final concentration was added to Mf−AO conjugate dissolved in 0.5 M NaCl (pH 7.5) at 2 °C. Mf−AO conjugate was collected as a precipitate by centrifugation and redissolved in 0.5 M NaCl (pH 7.5) three times to remove unreacted AO. The Ca-ATPase activity and the amount of binding AO were assayed after dialysis against 0.5 M NaCl (pH 7.5). 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 according to the method of Gomori (1942). The myofibrillar Ca-ATPase specific activity was expressed as nanomoles of P_i liberated per minute per milligram of protein. The protein concentration was determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard. The amount of AO bound to Mf was determined by the phenol−sulfuric acid method. Available lysine content in the protein was determined by 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).

Gel Filtration. Mf−AO mixture (1:1 w/w) containing 0.6 M sorbitol was incubated at 40 °C for 0, 12, and 24 h. After being solubilized in 0.5 M NaCl (pH 7.5) and centrifuged at 15000*g* for 30 min, the soluble fractions were applied to a gel filtration column (Sephacryl S-300, HiPrep-16/60, Amersham Pharmacia Biotech) pre-equliburated with the same buffer and eluted at 0.5 mL/min flow rate. Each 2.0 mL fraction was collected and assayed for protein and carbohydrate concentrations. The protein and AO concentrations in each fraction were determined according to the Lowry method (Lowry et al., 1955) and the phenol-sulfuric acid method respectively.

Electrophoretic Analysis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (1970) using 4 and 7.5% acrylamide slab gels for the stacking and resolving gels, respectively. An analysis sample was prepared as follows: 0.2 mL of the protein solution was added to a 0.8 mL of solution of 2% SDS, 8 M urea, and 2% β-mercaptoethanol with 20 mM Tris-HCl (pH 8.0) and heated in boiling water for 2 min. Each 10-μL sample

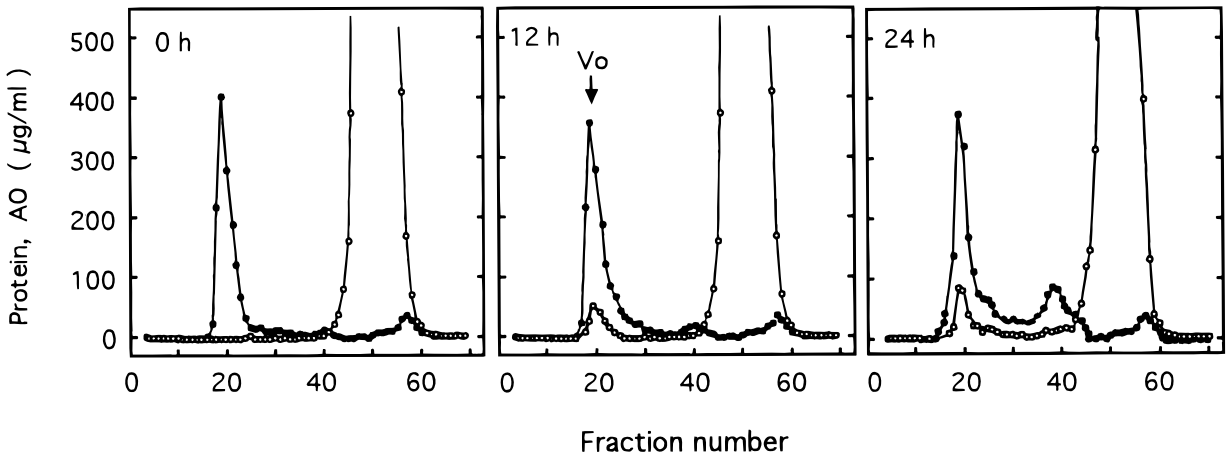

Figure 2. Elution patterns of Mf−AO mixture by gel filtration chromatography. Mf (○) and AO (●) concentrations were measured at each fraction. V_0 was a void volume of the gel filtration column.

was loaded on each gel lane, and Coomassie Brilliant Blue R was used for protein staining. Carbohydrate staining was performed using Alcian Blue 8GX (Wardi and Michos, 1972) for determining the binding component of AO.

RESULTS AND DISCUSSION

Conjugation of Mf with AO. Changes in total solubility in 0.5 M NaCl and Ca-ATPase activity of Mf during lyophilization and incubation were measured to investigate the protein denaturation during preparation of neoglycoprotein. Results are shown in Figure 1. When Mf−AO mixture (1:9 at weight ratio) containing 0.6 M sorbitol was lyophilized and incubated at 40 °C, although the total solubility and Ca-ATPase activity decreased during lyophilization, they were kept at high level after 24 h of incubation. On the other hand, in the case of Mf−AO mixture in the absence of sorbitol, the total solubility and Ca-ATPase activity were diminished to 48% of the intrinsic level only by lyophilization, and their rapid decreases were observed during the following incubation. These results indicate that AO itself has no protective effect on protein denaturation in the synthetic process of neoglycoprotein using the Maillard reaction. Thus, the addition of sorbitol is necessary to prepare Mf−AO conjugate with high solubility and to avoid protein denaturation.

To confirm the conjugation of AO with Mf, gel filtration chromatography and electrophoretic analysis were performed. Figure 2 shows the elution patterns of the 0.5 M NaCl-soluble fraction of Mf−AO mixtures incubated at 40 °C for 0, 12, and 24 h, respectively. The main protein peak identified as actomyosin by SDS−PAGE analysis eluted at the void volume and a large peak eluting at fractions of 41−61 were observed regardless of the incubation time. Furthermore, a new AO peak at the same elution volumes of actomyosin appeared in Mf−AO mixture incubated for 12 h, and it became larger when incubated for 24 h. Figure 3 shows the change in SDS−PAGE patterns of Mf−AO mixtures during incubation. In protein staining, the myosin heavy chain decreased markedly, and a broad band having lower mobility than the myosin heavy chain simultaneously appeared with the sample incubation. In addition, the broad myosin heavy chain bands were detected by carbohydrate staining. Results shown in Figures 2 and 3 indicate that AO was covalently bound to myosin heavy chains in Mf by incubating their mixture at 40 °C and 65% relative humidity. Furthermore, the broadening of the actin band and the disappearance of tropomyosin also occurred with an increase in the reaction time. Such changes in the SDS−PAGE pattern suggest the glycosylation of the protein subunits with AO. The loss of tropomyosin was also observed in the early stage of glycosylation with glucose and dextran, but a broad actin band was not observed in either synthetic system (Saeki, 1997; Fujiwara et al., 1998). These results suggest that lysine residues in tropomyosin would easily react with carbohydrates.

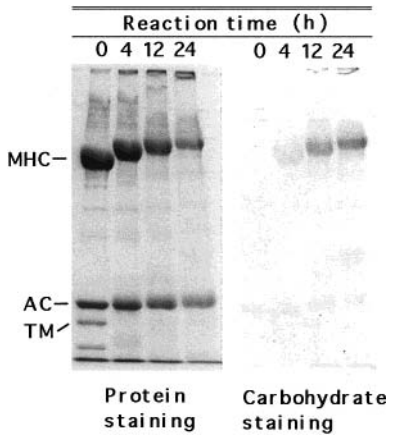

Figure 3. Changes in SDS−PAGE patterns of Mf during conjugation with AO. Mf was reacted with AO at 40 °C for 0−24 h. MHC, myosin heavy chain; AC, actin; TM, tropomyosin.

The amount of AO bound to Mf during incubation at 40 °C was measured at different mixing ratios of Mf and AO. As shown in Figure 4, Mf was rapidly conjugated with AO with a rise of AO concentration in the mixture. When Mf/AO was 1:0.4, 1:1, 1:4, and 1:9 and reacted for 24 h, the amounts of binding AO were 30, 71, 168, and 233 μg/mg of protein, respectively. Furthermore, it was confirmed that a small amount of AO reacted with Mf during lyophilization. The amounts of AO bound to Mf during lyophilization were 10 and 16 μg/mg of protein when Mf/AO was 1:4 or 1:9, respectively. The addition of sorbitol had no effect on the amount of AO bound to Mf (data not shown).

Changes in Total Solubility of Mf by Conjugation with AO. Changes in the total solubility of Mf in 0.16 and 0.5 M NaCl during conjugation with AO are shown in Figure 5. After just lyophilization, the total solubility of Mf in 0.5 M NaCl was 77 ± 2%. Although

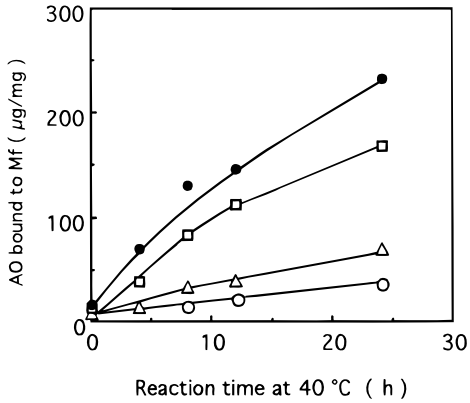

Figure 4. Effect of Mf−AO mixing rate on the amount of AO bound to Mf. Mf/AO = 1:0.4 (○), 1:1 (△), 1:4 (□), and 1:9 (●).

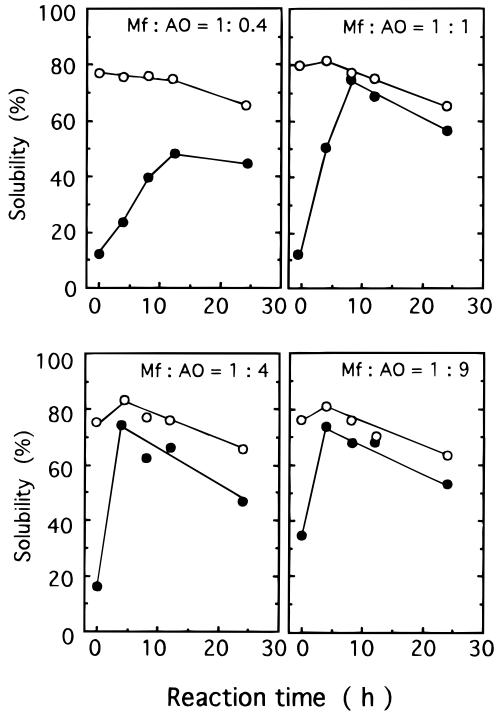

Figure 5. Changes in solubilities of Mf during conjugation with AO at different mixing ratios of Mf and AO; solubility in 0.16 M (○) and 0.5 M (●) NaCl at 4 °C and pH 7.5.

slight decreases in the total solubility were observed with the progress of the glycosylation, it remained 59−69% after reaction for 24 h. This change would be caused by thermally induced protein denaturation because the amount of AO bound to Mf has no effect on the decrease in the total solubility. On the other hand, the total solubility of Mf in 0.16 M NaCl increased with an increase in the amount of AO bound to Mf. After just lyophilization, the total solubility of Mf at 1:0.4 and 1:1 of Mf/AO was 11% and the total solubility increased markedly with the progress of the glycosylation. The highest values of the total solubility were 59% for 24 h (Mf/AO = 1:0.4) and 76% (Mf/AO = 1:1) for 8 h, when the amount of AO bound to Mf was 34 or 39 μg/mg of protein, respectively. As noted in Figure 4, when Mf was conjugated with AO during lyophilization at 1:4 and 1:9 of Mf/AO, the total solubility of the lyophilized Mf already reached 17 and 34%, respectively. In addition, their total solubility rapidly increased in contrast with the other two Mf−AO mixtures. They reached 74% at the highest values by reaction for 4 h, when the amount

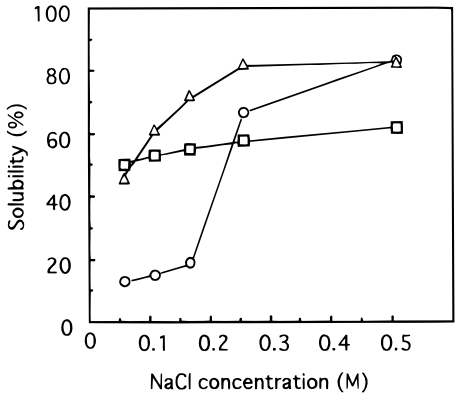

Figure 6. NaCl concentration dependence of solubility of Mf−AO conjugates. Mf−AO mixture (Mf/AO = 1:1) was incubated for 0 (○), 8 (△), and 24 (□) h.

of AO bound to Mf was 39 μg/mg of protein (Mf/AO = 1:4) or 70 μg/mg of protein (Mf/AO = 1:9). No increase was observed in the total solubility in 0.16 M NaCl when an Mf−sorbitol mixture without AO was incubated under the same conditions and the total solubility of Mf in 0.5 M NaCl slightly decreased, the same as Mf−AO conjugate. Therefore, the enhancement of the total solubility of Mf in 0.16 M NaCl would be caused by conjugation with AO. Figures 4 and 5 show that the significant change in the total solubility was obtained by conjugation with ∼3 wt % of AO. However, the improved total solubility in 0.16 M NaCl was impaired with the progress of the glycosylation. The loss of the total solubility would be caused by the protein denaturation because it occurred with a decrease in the total solubility in 0.5 M NaCl.

To investigate the change in NaCl concentration dependence of the total solubility of Mf, an Mf−AO mixture (Mf/AO = 1:1) and its conjugates prepared by reaction for 8 and 24 h were solubilized in 0.05−0.5 M NaCl solutions. Results are presented in Figure 6. In the Mf−AO mixture, the total solubility in 0.05−0.1 M NaCl solutions was <15%. A marked increase in the total solubility was observed in the range of 0.16−0.25 M NaCl, and a further increase was observed in the range of 0.25−0.5 M NaCl. On the other hand, the total solubility in 0.05 M NaCl was 43% when Mf−AO conjugate was prepared by reaction for 8 h and 36 μg/mg of AO was bound to Mf. Its total solubility increased gradually with a rise of NaCl concentration, and the highest value was observed in 0.25 M NaCl. Such an effect of glycosylation on the protein solubility in NaCl solutions was also observed in KCl solution. For instance, the total solubility in 0.16 M KCl also reached 74% by the reaction for 8 h (result not shown). Thus, it is apparent that the total solubility of Mf in a low ionic strength medium can be improved by conjugation with AO.

When the Mf−AO mixture was further reacted for 24 h (69 μg/mg of AO was conjugated), the total solubility in low ionic strength medium decreased with a decrease in the total solubility in high ionic strength medium, and the dependence of NaCl concentration for the total solubility of the Mf−AO conjugate almost disappeared. Therefore, excessive reaction, which exceeds protein heat denaturation, should be avoided in the preparation of Mf−AO conjugate with high solubility.

Lysine Loss of Mf with Improvement of Its Solubility. The improvement of the total solubility in low ionic strength medium was also observed when the

Table 2. Available Lysine Decrease Occurred with Improvement of Solubility in Low Ionic Strength Medium

glycosylation	reaction time[a] (h)	solubility in 0.16 M NaCl (%)	decreasing rate of lysine (%)	AO bound to Mf (μg/mg of protein)
Mf−AO conjugate[b]	0	10.8	0	
	8	74.0	4.2	36
	24	58.9	6.8	71
glucose modification[c]	0	11.9	0	
	12	65.4	16.7	
	24	52.3	28.2	

[a] Reactions were performed at 40 °C and 65% of relative humidity. [b] The weight rate of AO to Mf was 1:1. [c] Data from Saeki (1997).

10−20% of lysine in carp Mf was modified by glucose (Saeki and Inoue, 1997). On the other hand, as shown in Table 2, the loss of available lysine occurring with the glycosylation by AO was much lower than that of glucose modification when the same effect on the total solubility was obtained. Thus, it is apparent that the conjugation with AO is a superior manner for improving the water solubility of Mf in view of its nutritional aspect.

In conclusion, fish myofibrillar protein that can be solubilized in low and high ionic strength media was prepared by conjugation with AO using the Maillard reaction without significant loss of available lysine. Furthermore, it is apparent that suppression of protein denaturation is quite important for acquiring improved total solubility of Mf. A protectant having no reducing end carbonyl group such as sorbitol was useful to avoid protein denaturation during glycosylation. This procedure would be applicable to glycosylation of other thermally less stable proteins.

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Received for review January 29, 1999. Revised manuscript received October 19, 1999. Accepted October 28, 1999. A part of this research was supported by the Hokkaido Foundation for the Promotion of Scientific and Industrial Technology, Japan.

JF990081M