Improved Thermal Stability and Emulsifying Properties of Carp Myofibrillar Proteins by Conjugation with Dextran

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Carp myofibrillar proteins (Mf) were conjugated with dextran through the Maillard reaction, and their functional properties were investigated. Lyophilized myofibrils mixed with dextran (weight ratio 1:9) were kept at 40 and 50 °C (65% relative humidity) for preparing neoglycoprotein (Mf−Dex). The utilization of the protective effect of dextran was required for preparing Mf−Dex with high solubility, and the myosin heavy chain was selectively conjugated with dextran. Mf−Dex developed an excellent emulsifying property, and the solubility of Mf−Dex was almost unchanged by heating at 50 °C for 6 h. The improved emulsion stability was not impaired by heat treatment at 50 °C.

Keywords: *Fish; myofibrillar proteins; neoglycoprotein; glycosylation; dextran; solubility; thermal stability; emulsifying property; Maillard reaction*

INTRODUCTION

Fish meat is an abundant protein resource and is widely used as a material for processed foods. However, fish myofibrillar proteins are thermally and chemically less stable than that of other vertebrates (Yamashita et al., 1978; Hashimoto et al., 1982), and the excellent functional properties such as gel-forming ability, emulsifying ability, and water-holding ability are rapidly impaired with progress of protein denaturation (Kawashima et al., 1973; Numakura et al., 1989; Stefansson and Hultin, 1994). Therefore, many attempts have been made to stabilize myofibrillar proteins during processing and storage. For instance, edible cryoprotectants such as sorbitol and sucrose are often mixed with meat to prevent protein denaturation (Matsumoto and Noguchi, 1992). Rapid neutralization of meat by washing with alkaline solution is also used for suppressing acid denaturation of myofibrillar proteins in manufacturing fish meat products (Nonaka et al., 1990). Such information is significant for utilizing functional properties of fish muscle protein at an intrinsic level. However, there has been no attempt to improve the stability of fish myofibrillar proteins.

Recently, it has been reported that protein glycosylation with polysaccharides by the Maillard reaction was an effective means of improving the functional properties of food proteins (Kato et al., 1992; Nakamura et al., 1994; Matsudomi et al., 1995). Saeki (1997) and Saeki and Inoue (1997) also reported that some functional properties of fish myofibrillar proteins were improved by modifying lysine residues with glucose through the Maillard reaction. However, such modification has no effect on the thermal stability of fish myofibrillar proteins. In this study, the authors attempted to prepare neoglycoprotein from carp myofibrillar proteins by conjugation with dextran by the Maillard reaction and will describe the improved thermal stability and some functional properties of fish myofibrillar proteins.

MATERIALS AND METHODS

Materials. A cultured live carp (*Cyprinus carpio*) was obtained at a local fish market. Dextran (avg mol wt = 78 000) was purchased from Sigma Chemical Co. (St. Louis, MO), adenosine 5′-triphosphate (ATP) was from Pharmacia Biotech (Uppsala, Sweden), and corn oil was from Acros Organics Ltd. (Pittsburgh, PA). All other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Glycosylation of Myofibrillar Proteins with Dextran. Carp myofibrillar proteins were prepared from ordinary muscle by the method previously described (Saeki, 1997), suspended in 50 mM NaCl, and then mixed with dextran solutions. The final concentration of protein was adjusted to 6.0 mg/mL, and dextran was also adjusted to 6.0, 12.0, 24.0, or 54.0 mg/mL. A 5 mL amount of the mixtures placed into test tube (diameter = 16 mm) was frozen at −40 °C and immediately lyophilized in a freeze-dryer (FDU-506, Tokyo Rika Co., Ltd., Tokyo, Japan). The lyophilization was stopped when the sample temperature reached 15 °C. The lyophilized myofibrillar proteins (Mf) mixed with dextran thus obtained were immediately stored at −25 °C and used within 30 days of preparation. Glycosylation of Mf was performed through a controlled dry state according to a modification of the procedure of Kato et al. (1992). The lyophilized Mf mixed with dextran was incubated at 20−60 °C and 65% relative humidity in a humidity cabinet (Model PR-1G, Tabai Espec Corp., Tokyo, Japan). After reaction, Mf conjugated with dextran (Mf−Dex) at 4 °C was suspended in 30 mL of 0.1 M NaCl and 40 mM Tris-HCl (pH 7.5) and then centrifuged at 8000*g* for 20 min at 4 °C to remove unreacted dextran. Mf−Dex was collected as a precipitate after washing and centrifuging five times.

The protein concentration was determined by the biuret method (Gornall et al., 1949) using bovine serum albumin as a standard. The amount of dextran bound to myofibrillar proteins was determined by the phenol−sulfuric acid method (Dubois et al., 1956). Available lysine content in the protein 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

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Table 1. Comparison of Emulsifying Properties of Mf−Dex

specimen[a]	dextran bound to proteins (μg/mg)	emulsifying activity[b] (OD at 500 nm)	emulsifying stability[b] (min)
no reaction	0	1.67[a]	16.0[a]
50 °C/12 h	174	1.73[a]	29.4[b]
50 °C/24 h	293	1.78[a]	36.9[c]
50 °C/48 h	499	1.93[b]	99.4[d]
40 °C/24 h	98	1.68[a]	19.0[a]
40 °C/48 h	188	1.71[a]	26.6[b]
40 °C/72 h	281	1.88[a]	34.4[c]

[a] Relative humidity, 65%. [b] Different letters signify a significant difference ($n = 3$, $p < 0.05$).

final concentration) to remove Tris buffer and redissolved in 50 mM phosphate buffer (pH 9.5) containing 2% sodium dodecyl sulfate (SDS) using a homogenizer (Ultra-turrax T 25/N-8G, IKA-Labortechnik, Staufen, Germany) operating at 13 500 rpm for 2 min at 20−25 °C.

Solubility and Ca-ATPase Activity. Mf−Dex was dissolved in 0.5 M NaCl and 40 mM Tris-HCl (pH 7.5) at 2.0−5.0 mg/mL of the final protein concentration with a Potter-Elvehjem homogenizer at 120 rpm for 1 min and dialyzed against the same buffer for 16 h at 4 °C. Ten milliliters of the dialyzate was placed in a 15 mL tube and centrifuged at 15000*g* for 30 min at 4 °C. Solubility was expressed as percent of protein concentration in the supernatant with respect to that of the total protein solution before centrifugation. The supernatant was used as a sample for evaluating the thermal stability and emulsifying properties of Mf−Dex.

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 (1942). The myofibrillar Ca-ATPase specific activity was expressed as μmol Pi liberation/min·mg of protein.

Electrophoretic Analysis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed by the method of Laemmli (1970) using 4% and 7.5% acrylamide slab gels for the stacking and the resolving gels, respectively. An analysis sample was prepared as follows: 0.2 mL of the protein solution was added to an 0.8 mL 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 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 dextran.

Thermal Stability. Thermal stability was followed by monitoring changes in turbidity and solubility of the protein solution during heating. Lyophilized myofibrils mixed with dextran (weight ratio 1:9) were kept at 40 °C for 12, 24, and 48 h. Mf−Dex's containing 62, 110, and 196 μg/mg of dextran thus prepared were dissolved in 0.5 M NaCl. After the pH was adjusted to 6.2 or 7.5 by 40 mM Tris and 1 M HCl, each protein solution was stirred for 16 h at 4 °C and then centrifuged at 15000*g* for 30 min at 4 °C. The supernatant having the protein concentration adjusted to 2.0 mg/mL was placed in a 1-cm path cuvette covered with Parafilm (American National Can, Greenwich, CT), and the change in turbidity during heating at 50 °C for 0−6 h was measured at 500 nm. The pH of Mf−Dex solutions (pH 6.2 and 7.5) at 50 °C were changed to 5.6 and 6.9, respectively. The heat-treated protein solution was cooled in ice−water, and the solubility was measured in the manner described above. The pH of both protein solutions were recovered to previous values by cooling.

Emulsifying Properties. The emulsifying properties of Mf−Dex were determined according to the method of Pearce and Kinsella (1978). Mf−Dex's prepared at 40 and 50 °C (shown in Table 1) were dissolved in 0.5 M NaCl and 40 mM Tris-HCl (pH 7.5). The protein solution (2.1 mL, 3.0 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) operating at 13 500 rpm for 1 min in ice−water. After being allowed to stand for 1, 10, 20, 40, and 60 min at 4 °C, 0.15 mL of 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 in a 1-cm path cuvette. The emulsifying activity was expressed by the turbidity of the diluted emulsion after 1 min of standing. The emulsifying stability was expressed by measuring the time required for the turbidity of the diluted emulsion to be reduced to half its initial value. Pearce and Kinsella (1978) also reported that creaming occurs by removing oil globules from the bulk of the emulsion. Creaming that occurred in the emulsion was then observed with the naked eye. Furthermore, the thermal stability of the emulsifying properties of Mf−Dex was examined after heating at 50 °C for 3 h. 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.

Figure 1. Effect of dextran on glycosylation of Mf. Mf was reacted with different amounts of dextran at 40 °C for 24 h (○), 40 °C for 48 h (△), and 50 °C for 48 h (□).

RESULTS AND DISCUSSION

Conjugation of Mf with Dextran. Figure 1 shows the effect of the amount of dextran in the reaction system on glycosylation of Mf with dextran. The amount of dextran bound to Mf increased with the rise in the weight ratio of dextran to protein, and it reached a highest value when the ratio was 4:1. Figure 2 shows the effect of dextran on the solubility and Ca-ATPase activity of Mf−Dex's. Lyophilized Mf's containing different amounts of dextran were incubated at 40 °C for 72 h, 50 °C for 24 h, and 60 °C for 6 h. The amount of dextran bound to Mf was 288, 293, and 271 μg/mg of protein, respectively. In Figure 2, the data for Mf before and after lyophilization (unreacted with dextran) are also shown. As presented in Figure 2, although the solubility and the Ca-ATPase activity of Mf decreased with lyophilization, their losses were reduced by the addition of dextran. The losses of the solubility and the Ca-ATPase activity of Mf−Dex occurring with the reaction at 40 and 50 °C were also effectively diminished with an increase in the amount of dextran in the lyophilized mixture. When the weight ratio of dextran to Mf was 9:1, the losses were substantially reduced. These results indicate that the protein denaturation occurring with lyophilization and glycosylation was effectively depressed by dextran. It is important to use

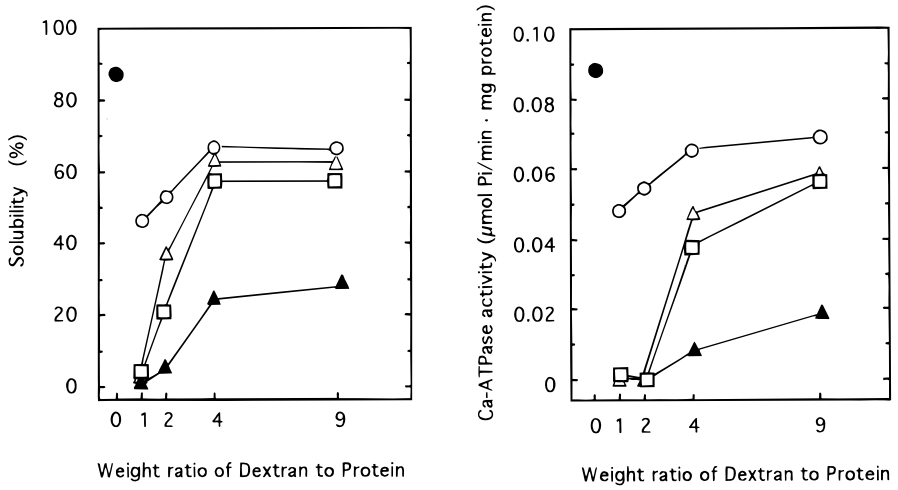

Figure 2. Protective effect of dextran on protein denaturation during lyophilization and glycosylation. Mf's (●) mixed with different amounts of dextran were lyophilized (○) and incubated at 40 °C for 72 h (△), 50 °C for 24 h (□), and 60 °C for 6 h (▲) for protein glycosylation.

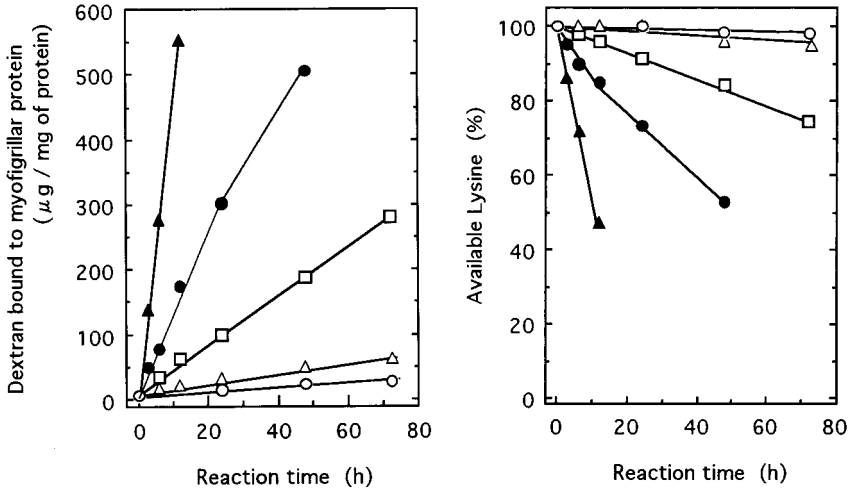

Figure 3. Effect of reaction temperature on glycosylation of Mf. Lyophilized Mf mixed with dextran (1:9) was incubated at 20 (○), 30 (△), 40 (□), 50 (●), and 60 °C (▲) for protein glycosylation.

the protective effect of carbohydrates when preparing neoglycoprotein from fish myofibrillar proteins.

Lyophilized Mf containing nine times the weight of dextran was incubated at different temperatures for conjugating with dextran. The amount of dextran bound to Mf and its available lysine content are shown in Figure 3. The amount of dextran bound to Mf increased with the rise in reaction temperature, and a rapid reaction was observed at more than 40 °C. In addition, the available lysine content decreased with an increase in the amount of dextran bound to Mf at any reaction temperatures. For instance, 9% and 17% of available lysine were lost when 100 and 200 μg of dextran were conjugated with one mg of Mf, respectively. However, as shown in Figure 2, lowering of the solubility of Mf−Dex was observed in the 60 °C reaction with a high concentration of dextran. Therefore, the authors determined that the mixing ratio of dextran with Mf should be 9:1 and the reaction should be performed at 40 and 50 °C as the conditions for preparing Mf−Dex with high solubility.

SDS−PAGE analysis was performed to determine the binding protein subunit with dextran because Mf contains many kinds of protein such as myosin, actin, and tropomyosin. As shown in Figure 4, the myosin heavy chain decreased markedly and a broad band having

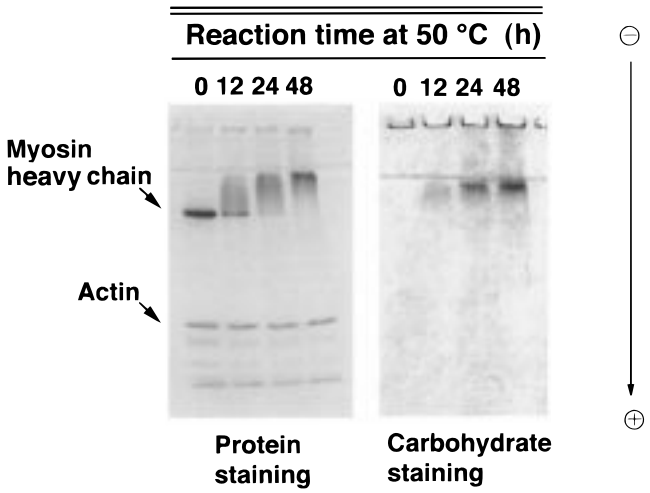

Figure 4. Changes in SDS−PAGE patterns of Mf during conjugation with dextran. Mf was reacted with dextran at 50 °C for 0−48 h.

lower mobility than the myosin heavy chain simultaneously appeared with the progress of glycosylation. Although a slight decrease in tropomyosin was observed, the other protein band remained practically unchanged. Furthermore, the broad bands that appeared with a

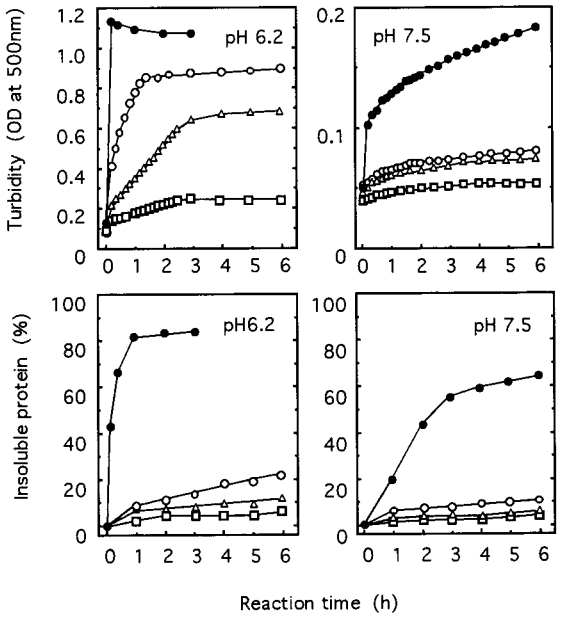

Figure 5. Effect of heat treatment on turbidity and solubility of Mf−Dex. Mf (●) and Mf−Dex containing 62 (○), 110 (△), and 197 (□) μg/mg of dextran were dissolved in 0.5 M NaCl (pH 6.2 and 7.5) and heated at 50 °C.

decrease in the myosin heavy chain were detected by carbohydrate staining. The same SDS−PAGE pattern was observed in the Mf−Dex obtained by reaction at different temperatures (data not shown). These results indicate that the dextran was selectively conjugated with myosin.

Thermal Stability of Mf−Dex. Unglycosylated Mf and Mf−Dex's containing different amounts of dextran were dissolved in 0.5 M NaCl solutions (pH 6.2 and 7.5) and then heated at 50 °C. Figure 5 shows the changes in the turbidity and solubility of Mf−Dex's and unglycosylated Mf during heating. At pH 7.5, the turbidity of unglycosylated Mf markedly increased, and 63% of the protein was insolubilized during heating for 6 h. On the contrary, when Mf−Dex's containing more than 62 μg/mg of dextran were heated, the turbidity slightly rose, and the amount of insolubilized protein was less than 10% of the total protein during heating for 6 h. Because fish myofibrillar proteins are unstable under acid conditions (Hashimoto and Arai, 1985), the protein solutions at pH 6.2 showed significant aggregation on heating. The turbidity of unglycosylated Mf rapidly increased and more than 80% of the proteins became insolubilized within 1 h. However, the solubility of Mf−Dex's containing 62 μg/mg and 110 μg/mg of dextran remained at a high value during heating, although turbidity increases occurred. In addition, the turbidity and the solubility of Mf−Dex containing 197 μg/mg of dextran were almost unchanged. The loss of solubility of unglycosylated Mf was not suppressed by the addition of dextran corresponding to 10 wt % of the protein (data not shown). Therefore, the results shown in Figure 5 indicate that the thermal stability of fish myofibrillar proteins is improved by conjugation with dextran.

The major protein conjugated with dextran was myosin as shown in Figure 4. In addition, the authors confirmed that the glycosylated myosin maintained its actin-binding ability because the viscosity of Mf−Dex solution at 10 °C decreased with the addition of 1 mM of ATP and 1 mM of $MgCl_2$, which indicates the dissociation of myosin−actin complexes (data not shown).

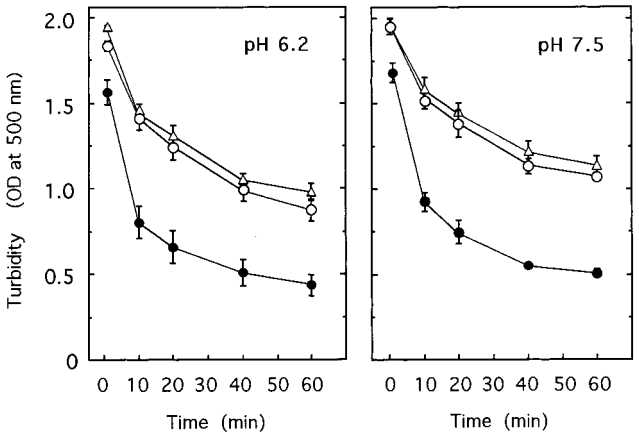

Figure 6. Thermal stability of emulsifying property of Mf−Dex: (●) Mf, (○) Mf−Dex containing 499 μg/mg of dextran, (△) heat-treated Mf−Dex. Values are mean ± standard deviation ($n = 3$).

Therefore, it is apparent that the glycosylation of myosin causes the improvement in the thermal stability of Mf. Thermal aggregation of myosin is likely hindered by attaching dextran.

Emulsifying Properties of Mf−Dex. Mf−Dex's containing 98−499 μg/mg of dextran were prepared by reaction at 40 and 50 °C, and their emulsifying properties were examined. The results are listed in Table 1. The emulsifying activity of Mf, although increased by conjugation with 499 μg/mg of dextran, was little affected by conjugation with dextran. On the other hand, the emulsion stability was effectively improved with an increase in the amount of binding dextran. When an emulsion of unreacted Mf and corn oil was kept at 4 °C, a cream layer was clearly formed within 8 min after emulsifying. In contrast, such a phase separation was not observed in the emulsion from Mf−Dex's containing 293 μg/mg and 499 μg/mg of dextran. These results also indicate that conjugation with dextran is an effective means of improving the emulsifying properties of fish myofibrillar proteins.

Because Mf gained greater thermal stability by conjugation with dextran as shown in Figure 5, the effect of heating on the emulsifying property of Mf−Dex was then investigated. Only 4.5% (pH 6.2) or 5.7% (pH 7.5) of total protein was insolubilized when Mf−Dex containing 499 μg/mg of dextran was dissolved in 0.5 M NaCl and 40 mM Tris-HCl (pH 6.2 or 7.5) and then heated at 50 °C for 3 h. After removal of insoluble protein by centrifugation, the heated Mf−Dex was emulsified with corn oil, and their emulsifying properties then measured. We did not investigate unglycosylated Mf because its solubility decreased to 16% and 32% at pH 6.2 and 7.5, respectively. As shown in Figure 6, the emulsifying activity of Mf−Dex was unchanged by the heat treatment under both pH conditions. In addition, there was no difference in the turbidity change of the emulsions between heat-treated and unheated Mf−Dex's ($p > 0.01$). Both heat-treated Mf−Dex's kept a high emulsifying stability. These results indicate that the heat treatments under both pH conditions have no effect on the improved emulsifying property of Mf−Dex.

In conclusion, the thermal stability and the emulsifying property of carp myofibrillar proteins were improved by conjugation with dextran through the Maillard reaction. Furthermore, the improved emulsifying stability of Mf was not impaired by heat treatment at 50

°C. The loss of available lysine is a nutritional problem in protein glycosylation by the Maillard reaction. For instance, more than 40% of lysine was lost when the emulsifying properties of Mf were improved by reaction with glucose (Saeki, 1997). However, in this study, when the thermal stability and the emulsifying property were improved by conjugation with dextran, the available lysine loss was 4.6% (conjugated 62 μg/mg of dextran) and 15.0% (conjugated 174 μg/mg of dextran), respectively. Therefore, protein glycosylation with dextran would be an effective method of improving the thermal stability of the functional properties of fish myofibrillar proteins.

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