Improved solubility and stability of carp myosin by conjugation with alginate oligosaccharide

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ABSTRACT: Carp myosin was conjugated with alginate oligosaccharide (AO) through the Maillard reaction under low relative humidity, and the functional properties of the myosin-AO conjugate were investigated to clarify the role of myosin in the functional improvement of fish myofibrillar proteins (Mf) by the glycosylation. The findings were as follows. First, myosin became highly solubilized at lower NaCl concentrations by conjugation with AO and NaCl-dependence of the solubility was lost when >12% of the available lysine residues were reacted with AO and 50 μg/mg of AO was attached to myosin. Second, the thermal stability of myosin was effectively improved by conjugation with AO. Heat-treatment at 50°C for 6 h has no effect on the solubility of the myosin-AO conjugate regardless of the NaCl concentration. Third, the improved functionalities of myosin conjugated with AO remained even at a nearly isoelectric point. The improving effect of AO-conjugation on the characteristics of myosin was almost the same as Mf reacted with AO. Therefore, it is apparent that the improved functionalities of the glycosylated Mf reflect the functional changes of myosin.

KEY WORDS: alginate oligosaccharide, glycosylation, isoelectric point, Maillard reaction, myosin, solubility, thermal stability.

INTRODUCTION

Fish and shellfish meats are important protein resources and are widely used as materials for processed seafood. The functional properties of the myofibrillar proteins (Mf), such as water-holding capacity, emulsifying ability, gel-foaming ability, and water-solubility are responsible for characteristics of fish products. However, fish Mf is thermally and chemically less stable than that of other vertebrates and the functional properties are readily impaired with a progress of protein denaturation under high temperature, low pH, and high salt concentration. Thus, many attempts to stabilize Mf during processing and storage have been carried out by controlling pH, temperature and salt concentration of food component.

Recently, it has been reported that the functional properties of fish and shellfish Mf have been improved by glycosylation using the Maillard reaction. Carp and scallop Mf became solubilized in a low-ionic strength medium by reaction with glucose and ribose. Emulsifying properties and thermal stability of carp Mf were also enhanced by conjugation with dextran. Additionally, it was reported that conjugation with alginate oligosaccharide (AO) using the Maillard reaction provides water-soluble Mf with high thermal stability and excellent emulsion-forming ability. These findings suggest that the neoglycoprotein synthetic system using the Maillard reaction would contribute to develop high utilization of fish meat proteins and AO is a superior material to obtain high-functional fish neoglycoprotein. However, there is little information about the molecular mechanism of the functional improvement of Mf by conjugation with AO.

It is known that the functional properties of fish meat as a food material reflect biochemical characteristics of myosin, which is the major protein component of Mf. For example, the stability of food functions of fish meat was closely related to the behavior of myosin. Therefore, understanding the dynamic behavior of myosin during conjugation with AO could help to clarify the molecular mechanism of the functional improvement by conjugation with AO. In this work, purified carp myosin was conjugated with AO and changes in the solu-
bility and the stability were investigated to compare with that of Mf.

MATERIALS AND METHODS

Materials

A cultured live carp *Cyprinus carpio* was obtained at a local fish market. Adenosinetriphosphate (ATP) and bovine serum albumin were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Acrylamide, Sodium docecylsulfate (SDS) and Coomassie Brilliant Blue R were obtained from Amersham Pharmacia Biotech (Upsala, Sweden). Fuchsin basic was purchased from Chroma-Gesellschaft Co. (Kongen, Germany). Sodium alginate from brown algae and all other chemicals (reagent grade) were from Kanto Chemical Co. Inc. (Tokyo, Japan).

Preparation of myosin

Carp ordinary muscle was washed and homogenized in 0.16 M KCl containing 20 mM Tris-HCl (pH 7.5), and further dissolved in 0.5 M KCl (pH 7.5). Another 2 mM ATP-Mg was added to dissociate the actomyosin complex. After addition of 40% saturation of ammonium sulfate, the protein solution was centrifuged at 30 000 ¥ g for 1 h to remove actin as a precipitate. Myosin in the supernatant was precipitated and collected by raising the ammonium sulfate saturation up to 55%, dissolved in 0.5 M NaCl containing 20 mM Tris-HCl (pH 7.5), and dialyzed against the same buffer to remove ammonium sulfate. After collected as a supernatant by centrifugation at 100 000 ¥ g for 1 h, purified myosin, thus obtained, was dialysis against 50 mM NaCl. All the steps in the preparation of myosin were carried out below 6∞C. The protein concentration was determined by the biuret method using bovine serum albumin as a standard.

Preparation of alginate oligosaccharide

AO was prepared by degrading sodium alginate using alginate lyase which was purified from the culture supernatant of *Pseudoalteromonas elyakovii* (IAM 14594). A total of 50 mM MgCl₂ and 1000 unit/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 (Type No.3, Advantec Toyo Co. Ltd, Tokyo, Japan), concentrated with a rotary evaporator, and then mixed with 80% ethyl alcohol. The AO, which has been collected as a precipitate by centrifugation at 10 000×g for 30 min, was resuspended in 80% ethanol to remove MgCl₂. This step was repeated five times. After being redissolved in distilled water, AO was ultrafiltrated using a polymer membrane (M.W. cut off limit: 50 000, Type Q0500, Advantec Toyo Co. Ltd, Tokyo, Japan) to remove undegraded alginate, and was then lyophilized using a freeze-dryer. The average degree of polymerization of AO was 6.1.

Glycosylation of myosin with alginate oligosaccharide

Purified carp myosin suspended in 50 mM NaCl was mixed with AO and sorbitol. The protein and AO were adjusted to 5 mg/mL at final concentrations and a final concentration of sorbitol was adjusted to 0.6 M. Another 5 mL- aliquots of the myosin-AO mixture placed into test tubes were frozen at −40∞C and lyophilized in a freeze-dryer. The lyophilization was stopped when the sample temperature reached 15∞C. The lyophilized samples were stored at −25∞C and used within 30 days of the preparation. Myosin-AO conjugate was prepared by using the Maillard reaction between ε-amino groups of lysine in myosin and reducing end-terminus of AO under controlled temperature and relative humidity conditions. The lyophilized myosin-AO mixtures described above were incubated at 40∞C and 35% relative humidity for 0–48 h in a humidity cabinet (model PR-1G, Tabai Espec Co., Tokyo, Japan). The myosin-AO conjugate, thus obtained, was immediately dissolved in various solutions by the following process.

Electrophoretic analysis

The glycosylated samples were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmlli using 4% and 7.5% acrylamide slab gels for the stacking and resolving gels, respectively. Coomassie Brilliant Blue R was used for protein staining. Carbohydrate staining was performed using Fuchsin basic to determine the binding of AO.

Available lysine content

The myosin-AO conjugate was dissolved in 0.5 M NaCl containing 40 mM Tris-HCl (pH 7.5), and available lysine content in the protein was deter-
mined by spectrophotometric analysis using o-
phthalaldehyde and N-acetyl-L-cysteine.17

Before the analysis of available lysine, the pro-
tein was precipitated with 7.5% trichloroacetic
acid (at the final concentration) to remove the
Tris buffer and was redissolved in 50 mM phos-
phate buffer (pH 9.5) containing 2% SDS. The
assays were performed within 48 h of the protein
glycosylation.

Amount of alginate oligosaccharide bound
to myosin

A total of 60% of saturated ammonium sulfate at
the final concentration was added to the myosin-
AO conjugate dissolved in 0.5 M NaCl containing
40 mM Tris-HCl (pH 7.5) at 2°C. The myosin-AO
conjugate was collected as a precipitate by centri-
fugation at 15 000×g for 30 min and redissolved in
0.5 M NaCl (pH 7.5) to remove unreacted AO. This
step was repeated three times. The amount of AO
bound to myosin was determined by the phenol-
sulfuric acid method.18

Solubility

The myosin-AO conjugate was dissolved in 0.1 M
and 0.5 M NaCl containing 40 mM citrate-NaOH
(pH 3.5–5.0), 40 mM Tris-maleate- NaOH (pH 5.5–
6.5), and 40 mM Tris-HCl (pH 7.5) at 1.0 mg/mL of
the final protein with a homogenizer (Ultra-turrax
T25/N-8G, IKA-Labohnechnik, Staufen, Germany)
operating at 13 500 rpm for 60 s. Samples were
immediately centrifuged at 15 000×g for 30 min at
4°C. The supernatant and the total protein solution
before centrifugation were mixed with an equal
volume of 2.0 M NaOH, and their protein concen-
trations were determined by the Bradford
method19 using bovine serum albumin as a stan-
dard. The solubility was expressed as the ratio (%)
of protein in the supernatant to that of the total
protein solution. NaCl concentration dependency
of myosin-AO conjugate was also examined by
measuring the solubility in 0.1–0.5 M NaCl (pH 7.5)
in this study. In the preliminary experiment, we
confirmed that unreacted AO and sorbitol have no
effect on the solubility of myosin-AO conjugate.

Isoelectric point

The isoelectric point (pI) of carp myosin was esti-
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Isoelectric point

The isoelectric point (pI) of carp myosin was esti-
mated with DNA/Protein analytical software (DNA-
sis, version 3.5, Hitachi Software Co. Ltd, Tokyo,
Japan). The primary structure of carp myosin20 was
used to estimate the pl of the proteins.

Thermal stability

The thermal stability of the myosin-AO conjugate
was examined by monitoring the solubility change
by heat treatment. After the removal of unreacted
AO and sorbitol by the ammonium sulfate fraction-
ation (60% saturation), the myosin-AO conjugate
was dissolved in 0.1 M and 0.5 M NaCl solutions
containing 40 mM Tris-maleate-NaOH (pH 6.2) or
40 mM Tris-maleate (pH 7.5), and centrifuged at
20 000×g for 30 min to take out possible debris.
Another 1 mL- aliquots of the myosin-AO conjugate
(2 mg/mL) were introduced in polyethylene tubes,
sealed with Parafilm (Pechiney Plastic Packaging
Inc., Menasha, Wisconsin, USA), and heated at
50°C up to 6 h with a thermostatic water-bath. The
pH of the myosin solutions containing 40 mM Tris-
maleate (pH 7.5) were shifted to 6.9 during heating
at 50°C and returned to 7.5 after cooling. The pH of
the myosin solutions containing 40 mM Tris-male-
ate (pH 6.2) was not affected by heating. After
being cooled in ice-water, the heated myosin-AO
conjugates were centrifuged at 20 000×g for 30 min
at 4°C. The solubility was expressed as the ratio (%)
of protein concentration before and after heating.
The protein concentration was determined by the
Lowry method.21

RESULT AND DISCUSSION

Reaction of myosin with alginate oligosaccharide

Figure 1 shows the changes in SDS-PAGE patterns
of the myosin-AO mixture during incubation at
40°C and 35% relative humidity. Although no degrada-
tion and polymerization were observed in the
myosin heavy chain, its electrophoretic mobility
decreased gradually with elapse of the incubation
time. The reduction of the electrophoretic mobility
has previously been observed in Mf reacted with
monosaccharides.5,7 Additionally, the myosin
heavy chains were detected by carbohydrate stain-
ing when incubated for more than 6 h. Figure 2
shows the progress of the Maillard reaction
between myosin and AO. A rapid loss of the avail-
able lysine occurred and 10% of the total lysine
residues decreased during the reaction for 6 h. The
loss of the available lysine was observed subse-
quently and it decreased to 76% of the initial value
after the reaction for 48 h (Fig. 2a). The amount of
AO bound to myosin increased simultaneously
with the loss of the available lysine. The binding AO
increased markedly during the reaction for 6 h and
it reached 84 μg/mg after the reaction for 48 h.
A strong correlation between the binding AO
(μg/mg) and the lysine loss (%) was observed as
presented in Fig. 2b. Indeed, 1% of the total available lysine was lost when 3.7 μg/mg of AO was bound to myosin. The results of Figs 1 and 2 conclude that AO was covalently attached to myosin heavy chain through the Maillard reaction between the ε-amino group of the lysine of the protein and the reducing terminus of AO.

Generally, the formation of the brown coloration is observed with the progress of the Maillard reaction. Although no browning was observed in myosin-AO conjugate as presented in Fig. 2 (data not shown), a marked brown coloration of myosin-AO conjugate occurred by heating for 72 h. This result indicates that the Maillard reaction between myosin and AO was controlled at the early stage in the neoglycoprotein synthetic system.

Improved solubility of myosin in a low ionic strength medium by conjugation with alginate oligosaccharide

Figure 3a shows changes in the solubility of myosin in 0.1 M and 0.5 M NaCl (pH 7.5) during the reaction with AO. The solubility of myosin in 0.5 M NaCl was 94.3% and it remained unchanged during the reaction for 48 h. This result indicates that the conjugation with AO has little effect on the intrinsic solubility of myosin. In contrast, the
solubility in 0.1 M NaCl increased markedly with the progress of the reaction and equaled the solubility in 0.5 M NaCl after 12 h of the reaction. No increase was observed in the solubility in 0.1 M NaCl, when the same amount of AO was added to myosin-sorbitol mixture after incubation under the same condition (data not shown). Therefore, it is apparent that the enhancement of the solubility of myosin in 0.1 M NaCl was caused by the conjugation with AO.

As presented in Fig. 3b, the NaCl concentration dependency of the solubility of myosin disappeared with an increase in the amount of the binding AO, and it completely disappeared when the solubility in 0.1 M NaCl reached the maximum value. It is known that myosin molecules spontaneously assemble into insoluble filaments in solutions of physiological ionic strength at neutral pH, and the NaCl concentration dependency of the solubility of native myosin reflects the filament-forming ability. Indeed, the filament-forming ability of myosin was impaired by the reaction with monosaccharides. Therefore, the AO bound to myosin may also affect the filament formation in a low ionic strength medium.

The amount of AO bound to myosin and the reacted lysine residues were plotted against the solubility in 0.1 M NaCl (Fig. 4). It is confirmed that the solubility improvement was performed while 12.5% of the available lysine were lost by the reaction with AO (Fig. 4a) and 40 μg/mg of AO was attached to myosin (Fig. 4b). Additionally, the further progress of the reaction has no effect on the solubility change. Tanabe and Saeki reported that about 30% of the available lysine loss was required to improve the carp myosin solubility by the reaction with monosaccharides. Therefore, the glycosylation with AO seems to be an effective manner to improve the solubility of fish myosin.

![Fig. 4](image-url)  
Relation between solubility of myosin and lysine residues reacted with alginate oligosaccharide (AO). The rate of lysine residues reacted with AO (a) and the amount of AO bound to myosin (b) were plotted against the solubility in 0.1 M NaCl.

![Fig. 5](image-url)  
Effect of pH on solubility of myosin conjugated with alginate oligosaccharide (AO). Myosin conjugated with 84 μg/mg of AO was dissolved in 0.1 M NaCl (△) and 0.5 M NaCl (○) at pH 3.5–7.5. Native myosin (●) was also examined as a control.

### Solubility of myosin conjugated with alginate oligosaccharide as affected by pH

Effect of pH on the solubility of the myosin-AO conjugate containing 84 μg/mg of AO was measured and compared with that of native myosin. The result is shown in Fig. 5. The solubility of native myosin in 0.5 M NaCl was gradually diminished in the pH range of 5.5–7.5 and it became insoluble below pH 5.0. The pl value of carp myosin estimated from the amino acid composition was pH 5.52. Therefore, the solubility decrease of native myosin at nearly the pl could be caused by the loss of net charge of the protein. In contrast, the myosin conjugated with AO showed high solubility (>90%) at the range of pH 5.5–7.5, regardless of NaCl concentration. In addition, the solubility at pH 5.0 showed 50.0% in 0.1 M NaCl and 77.2% in 0.5 M NaCl.
Myosin conjugated with oligosaccharide  FISHERIES SCIENCE  901

It is sure that the pI of myosin shifts to the acidic side because the positively charged lysine residue was lost by the reaction with AO. Indeed, the pI of the carp myosin conjugated with 84 mg/mg AO (the available lysine loss was 24%) was estimated to be 4.88. Therefore, the pI shift to a more acidic value by conjugation with AO could contribute to the solubility improvement of myosin in the pH range more than 5.0.

Thermal stability of myosin by conjugation with alginate oligosaccharide

Figure 6 shows the solubility change of the myosin caused by heating. Native myosin and myosin-AO conjugates containing 14–84 µg/mg AO were dissolved in 0.1 M and 0.5 M NaCl (pH 6.2 and 7.5) and heated at 50°C for 6 h. The solubility of native myosin in 0.5 M NaCl solutions at pH 6.2 and 7.5 decreased to 18% and 37%, respectively. In contrast, the solubility of the myosin-AO conjugates in the same buffer remained unchanged during heating for 6 h. Furthermore, it was confirmed that the heat treatment has no effect on the solubility of another myosin-AO conjugate regardless of NaCl concentration or pH, except the myosin-AO conjugate containing 14 µg/mg AO in 0.5 M NaCl (pH 6.2). In the preliminary experiment, the addition of an equal amount of AO has no protective effect on the protein solubility under all heating conditions (data not shown). Therefore, the result in Fig. 6 indicates that the myosin-AO conjugates have higher stability in a wide range of NaCl concentrations, pH levels, and thermal conditions than native myosin.

The results of this work showed clearly that the solubility in low ionic strength media and the thermal stability of purified myosin were effectively improved by conjugation with AO, and it was the same improving effect observed in Mf.9,10 Therefore, it is apparent that improved functionalities of the glycosylated fish Mf reflect the functional changes of myosin.

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REFERENCES


