Characterization of Fish Myofibrillar Protein by Conjugation with Alginate Oligosaccharide Prepared Using Genetic Recombinant Alginate Lyase

RYO SATO, TOMOO SAWABE, AND HIROKI SAEKI

ABSTRACT: The production of alginate lyase using genetically modified *Escherichia coli* was superior to the purification of alginate lyase from a culture medium of *Pseudoalteromonas elyakovii* regarding production efficiency. When alginate oligosaccharide (AO) prepared using genetic recombinant alginate lyase was introduced to fish myofibrillar proteins, the protein obtained high water solubility and improved thermal stability, similarly to AO prepared using wild-type lyase. Therefore, the use of genetic recombinant technology for the production of alginate lyase would be useful for the functional improvement of fish myofibrillar proteins by conjugation with AO.

Keywords: fish protein, alginate oligosaccharide, genetic recombinant alginate lyase, solubility, thermal stability

Introduction

Protein glycosylation using the Maillard reaction is an effective method for improving the functional properties of food protein such as ovalbumin (Kato and others 1995), phosvitin (Nakamura and others 1998), lysozyme (Nakamura and others 2000), and β-lactoglobulin (Aoki and others 1997). Food functionalities, such as emulsion-forming ability, antioxidant activity, and antimicrobial activity, have been improved by conjugation with reducing sugars. In many attempts to improve the food functionalities of protein from fish flesh, the authors (Sato and others 2000, 2003) succeeded in the production of water-soluble myofibrillar protein (Mf) with high thermal stability and excellent emulsion-forming ability by conjugation with alginate oligosaccharide (AO). The new technology for giving water solubility to Mf with high stability and without protein degradation could contribute to the development of a new application of fish meat as a material of protein-rich liquid foodstuff. The AO for the protein modification was prepared from Lessonia (*Phaeophyta*) alginate using an alginate lyase from the marine bacterium *Pseudoalteromonas elyakovii* (Sawabe and others 1992). Therefore, the Mf-AO conjugate could contribute to the complex utilization of marine bioresources of fish protein, seaweed polysaccharide, and marine bacterial enzyme (Sato and others 2000).

The reducing-end terminus of AO can be retained completely at the enzymatic degradation using the alginate lyase (Sato and others 2000), and the Mf-AO conjugate can be prepared effectively by reacting the reducing-end terminus of AO with the ε-amino group of Mf. However, the purification of the alginate lyase from the *P. elyakovii* culture supernatant is not a highly productive step of the manufacture of the Mf-AO conjugate because several chromatography steps were difficult to implement in the culture supernatant, which includes seawater (Sawabe and others 1992). The enzyme production of *P. elyakovii* is also less sufficient than that of currently known commercially available enzymes produced by microorganisms.

Genetic recombinant technology is now being used to improve the productivity of valuable proteins. The alginate lyase gene of *P. elyakovii* is cloned and expressed in *Escherichia coli* cells (Sawabe and others 2001). The application of genetically modified *E. coli* would have a great advantage in the purification efficiency and yield of the recombinant alginate lyase due to (1) the use of a low-sodium chloride content in the medium for the cultivation of *E. coli*, (2) the availability of an affinity tag in the expressed enzyme, and (3) the densely packed form of the expressed enzyme in *E. coli* cells. However, changes in the biochemical traits in genetically modified enzymes expressed in *E. coli* have been known to occur in some cases (Old and Primrose 1994; Sambrook and Russell 2001). Before genetically modified alginate lyase can be applied, the ability of the AO produced by the enzyme to make the fish-myofibrillar-glycoprotein should be characterized.

In this study, alginate lyase was prepared using a genetically modified *E. coli* and the production efficiency was compared with the purification from a culture medium of *P. elyakovii*. In addition, the AO prepared by the genetic recombinant alginate lyase was introduced into Mf using the Maillard reaction, and the water solubilization of Mf and its thermal stability were examined and compared with the case of AO prepared using alginate lyase from *P. elyakovii*.

Materials and Methods

Materials

Sodium alginate from brown algae was purchased from Kanto Chemicals Co., Inc. (Tokyo, Japan). D-mannuronolactone was pur-

MS 20040226 Submitted 4/10/04, Reviewed 6/21/04, Accepted 8/15/04. *Authors are with Graduate School of Fisheries Sciences, Hokkaido Univ., Minato 3, Hakodate, Hokkaido 041-8611, Japan. Direct inquiries to author Saeki (E-mail: saeki@fish.hokudai.ac.jp).*

Published on Web 12/22/2004

chased from Funakoshi Co., Ltd. (Tokyo, Japan). A cultured live carp (*Cyprinus carpio*) was obtained at a local fish market. Bovine serum albumin (fraction V) was obtained from Merck Co. Ltd. (Darmstadt, Germany). All other chemicals (reagent grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of wild-type alginate lyase

The preparation of wild-type alginate lyase (W-lyase) was performed according to the method described by Sawabe and others (1992) with a slight modification. *P. elyakovii* isolated from decayed thalli of *Laminaria japonica* was cultured in 2700 mL of 75% seawater containing 0.8% casitone and 0.1% sodium alginate at 25 °C for 72 h with reciprocal agitation. The culture medium of *P. elyakovii* was then centrifuged at 20000 × *g* for 30 min, and the supernatant containing the extracellular lyase was used as the starting material for the purification of the enzyme. All ensuing operations were carried out at 0 to 4 °C. The culture supernatant was concentrated by ultrafiltration using a regenerated cellulose membrane (Type PLCCC, 5000 MW cutoff, Millipore) at less than 8 °C. The concentrated enzyme was dialyzed overnight against 0.1 M Tris-HCl (pH 7.5) and centrifuged at 20000 × *g* for 30 min to remove insoluble materials, and the supernatant was concentrated up to 10 times using a freeze-dryer overnight. Ten milliliters of the crude enzymes thus obtained was subjected to gel-filtration chromatography using Toyopearl HW-50 fine-grade column (Tosoh Co. Ltd., ϕ 2.6 × 90 cm, bed volume: 367 mL, void volume: 95 mL) equilibrated at 0.1 M Tris-HCl (pH 7.5) (flow rate: 0.5 mL/min). Each 5.0 mL fraction was collected and assayed for the alginate lyase activity by a method described later, and the fractions with alginate lyase activity (elution volume: 185 to 230 mL) were collected as W-lyase and stored at –70 °C until use.

Preparation of genetic recombinant alginate lyase

Genetic recombinant alginate lyase (G-lyase) was prepared according to the method of Sawabe and others (2001). The genomic DNA of *P. elyakovii* IAM 14594 was isolated using a Promega Wizard™ genomic DNA extraction kit according to the manufacturer's instruction. Recombinant plasmids were constructed according to Sambrook and others (1989). The genomic DNA of *P. elyakovii* was completely digested with *Hin*dIII, and the fragment of 2.7 kb thus obtained was inserted into the *Hin*dIII site of the pUC 118 and transformed into the *Escherichia coli* JM 109 strain. This clone, the pTPB 24 strain, was cultured in 200 mL of an LB broth containing 50% artificial seawater, 100 μg/mL ampicillin, and 5 mM isopropyl-β-D-thiogalactoside for 30 h at 30 °C. After collecting by centrifugation at 10000 × *g* for 15 min, the cell pellet was washed twice with 30 mM NaCl containing 10 mM Tris-HCl (pH 7.3), suspended in 200 mL of cold 20% (w/v) sucrose containing 1 mM EDTA and 50 mM Tris-HCl (pH 7.4), and subsequently incubated for 10 min at 4 °C with gentle agitation. The pellet was then centrifuged at 10000 × *g* for 15 min, resuspended in 50 mL of cold 5 mM $MgCl_2$ solution for 10 min at 4 °C with gentle agitation, and centrifuged at 14000 × *g* for 15 min. The supernatant, including a periplasmic fraction of the pTPB 24 strain, was used as G-lyase.

Measurement of lyase activity

When sodium alginate is degraded with alginate lyase, unsaturated uronic acid with a double bond between C_4 and C_5 was produced at the cutting-off site (Gacesa 1987), and the double bond showed a maximum absorbance at 235 nm (Tsujino and Saito 1962). The enzymatic activity of alginate lyase was measured by monitoring the increase in absorbance at 235 nm with a 1-cm-pass cuvette. The reaction mixture containing 0.1% sodium alginate, 0.1 M Tris-HCl (pH 7.5), and alginate lyase was incubated at 30 °C for 30 min. One unit of activity was defined as an increase in the absorbance of 0.010 per min (Sawabe and others 1992).

Preparation of alginate oligosaccharide

Alginate lyase (1000 units/L) was added to autoclaved 3% (w/w) sodium alginate containing 50 mM $MgCl_2$ and stirred at 30 °C for 96 h. The generation of alginate oligosaccharide (AO) was detected by measuring the absorbance at 235 nm of the reaction mixture after diluting 120 times. After degradation, the AO solution was centrifuged at 20000 × *g* for 30 min, and the supernatants were concentrated with a vacuum rotary evaporator (20 to 25 °C/60 kPa) and then mixed with 80% ethyl alcohol. The precipitated AO was collected by centrifugation at 20000 × *g* for 30 min and then resuspended in 80% ethyl alcohol. This step was repeated 5 times to remove $MgCl_2$. After being redissolved in distilled water, the AO was ultrafiltrated using a polymer membrane (MW cutoff limit = 50 kDa, Advantec Toyo Co., Ltd. Tokyo, Japan) to remove undegraded alginate and then lyophilized using a freeze-dryer. The yield of AO in the production step averaged 86%.

For this paper, the AO prepared with W-lyase and G-lyase was expressed as WAO and GAO, respectively. Five milligrams of WAO and GAO was applied to Cellulofine GCL-25m column (ϕ 2.6 cm × 70 cm, Seikagaku Kogyo Co., Ltd. Tokyo, Japan) eluted with 0.1 M Na_2SO_4 at 30 mL/h, and each 3.0 mL fraction was collected. The molar concentration (A) of each AO was measured by the phenol-sulfuric acid reaction (Dubois and others 1956) using D-mannuronolactone as a standard. The molar concentration of the reducing-end group (B) in each AO was determined by the Somogyi method (Somogyi 1952). The degree of polymerization of each AO was calculated as A/B (Sato and others 2000).

Preparation of the Mf-AO conjugate

Carp Mf was prepared from the ordinary muscle of a cultured live carp by the method of Saeki (1997), suspended in 50 mM NaCl containing 0.6 M sorbitol, and subsequently mixed with WAO or GAO. Both of the final protein and AO concentrations of Mf-AO mixtures were 6 mg/mL. The protein concentration was measured by the Biuret method (Gornall and others 1949) using bovine serum albumin as a standard. Five milliliters of the mixture was placed in a test tube (dia = 16 mm), frozen at –40 °C for 1 h, and immediately lyophilized using a freeze-dryer. The lyophilized Mf-AO mixtures were incubated at 40 °C or 50 °C under 35% relative humidity for 0 to 120 h in a temperature- and humidity-controlled chamber (model PR-1G, Tabai Espec Corp., Tokyo, Japan), and AOs were introduced into Mf through the Maillard reaction. The thus obtained Mf conjugated with WAO (Mf-WAO) and GAO (Mf-GAO) was immediately dissolved in 0.05 M and 0.5 M NaCl containing 40 mM Tris-HCl (pH 7.5) by the following process.

Available lysine content

Mf-GAO and Mf-WAO conjugates were dissolved in 0.5 M NaCl containing 40 mM Tris-HCl (pH 7.5), and the available lysine content in the protein was determined by spectrophotometric analysis using *o*-phthalaldehyde and *N*-acetyl-L-cysteine (Medina-Hernandez, Guarcia-Alvarez-Coque 1992). Before the analysis of available lysine, the protein was precipitated with 7.5% trichloroacetic acid (at the final concentration) to remove the Tris buffer and redissolved in a 50 mM phosphate buffer (pH 9.5) containing 2% sodium dodecyl sulfate (SDS). Each value of the available lysine in Figure 5 is the mean of 3 replicates that were reproducible within 4%.

Amount of AO bound to Mf

Sixty percent of saturated ammonium sulfate at the final con-

centration was added to Mf-GAO and Mf-WAO conjugates dissolved in 0.5 M NaCl containing 40 mM Tris-HCl (pH 7.5) at 2 °C. Mf-GAO and Mf-WAO conjugates were collected as a precipitate by centrifugation and redissolved in 0.5 M NaCl (pH 7.5) 3 times to remove unreacted AO. The amount of AO bound to Mf was determined by the phenol-sulfuric acid method (Dubois and others 1956). Each value of the binding AO to Mf in Figure 5 is the mean of 3 replicates that were reproducible within 5%.

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 0.8 mL of a 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 of sample was loaded on each gel lane, and Coomassie Brilliant Blue R was used for protein staining. Carbohydrate staining (periodic acid–Schiff staining) was performed according to the method of Zacharius and others (1969).

Measurement of solubility

Mf-WAO and Mf-GAO conjugates were dissolved in 0.05 M and 0.5 M NaCl containing 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-Laboatechnik, 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 before centrifugation were mixed with an equal volume of 2.0 M NaOH, and their protein concentrations were determined by the Bradford method (Bradford 1976) using bovine serum albumin as a standard. The solubility was expressed as the ratio (%) of protein in the supernatant to that of the total protein solution. In a preliminary experiment, we confirmed that unreacted AO and sorbitol have no effect on the solubility of Mf-WAO and Mf-GAO conjugates.

Estimation of thermal stability

The thermal stability of the Mf-AO conjugate was examined by monitoring the solubility change by heat treatment. After the removal of unreacted AO and sorbitol by ammonium sulfate fractionation, Mf-AO conjugates (2.0 mg/mL) were dissolved in 0.05 M and 0.5 M NaCl solutions containing 40 mM Tris-maleate (pH 7.5). The soluble fraction of Mf-AO conjugats obtained by centrifugation for 15000 × *g* for 30 min at 4 °C were immediately heated at 80 °C for 2 h with a water bath. After being cooled in ice water, the heat-treated Mf-AO conjugates were centrifuged at 15000 × *g* for 30 min at 4 °C. The solubility was expressed as the ratio (%) of protein concentration before and after heating.

Results and Discussion

Production efficiency of W-lyase and G-lyase

Figure 1 shows the procedures for the preparation of alginate lyases. W-lyase was secreted in the culture medium of *P. elyakovii*. When *P. elyakovii* was cultured in 2700 mL of the culture medium, 6640 units of W-lyase were obtained in the culture supernatant. W-lyase was recovered in 47% yield (total activity = 3120 units) at a purification process that included concentration and desalting. Consequently, it was estimated that 1126 units of W-lyase could be obtained from 1000 mL of the cultivated *P. elyakovii*. In the case of G-lyase, the alginate lyase gene was expressed in the *E. coli* pTPB 24 strain, and the produced G-lyase was mainly distributed in the periplasmic fraction (Sawabe and others 2001). After the purification process, 50 mL of the G-lyase solution having 13.1 unit/mL (total activity = 655 units) was purified from 200 mL of the gene-recombinant *E. coli*. This output corresponded to 2.9 times in the case of W-lyase, and the purification of G-lyase from the periplasmic fraction was performed as a simple procedure different from the purification of W-lyase from the culture medium described in the Materials and Methods section. These results indicate that the application of genetically modified *E. coli* is effective for the production of alginate lyase.

Figure 2 shows degradation of sodium alginate by the 2 types of lyase. The same time course of the generation of a double bond in the cutting-off site of alginate was observed when 1000 units /L of the G-lyase or W-lyase were added to a 3% sodium alginate solution. The degrees of polymerization of GAO and WAO obtained after a reaction for 96 h were 6.5 and 6.1, respectively. Furthermore, as shown in Figure 3, there was no difference in the molecular weight distribution between GAO and WAO. The degrees of polymerization of peaks I, II, and III in the chromatographs were determined as 7, 6, and 4 to 5, respectively. Alginates are linear (1 → 4)-linked glycu-

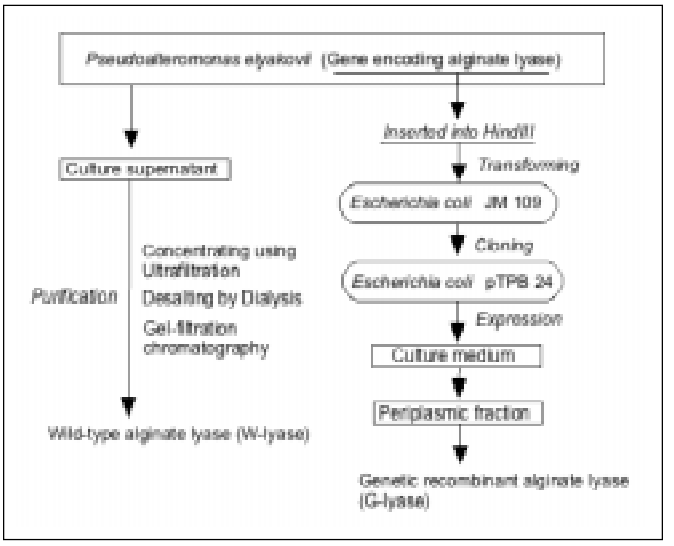

Figure 1—Procedure for production of alginate lyase.

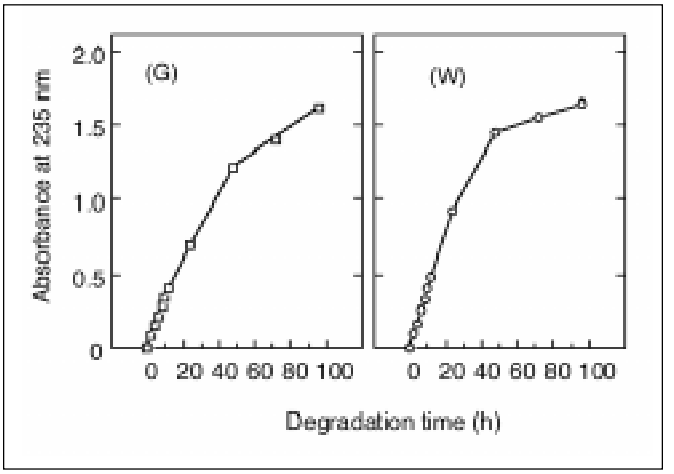

Figure 2—Degradation of sodium alginate by G-lyase (G) and W-lyase (W)

ronans comprised of residues of β-D-mannosyluronic acid (M) and its C-5 epimer α-L-gulusyluronic acid (G). These residues are arranged in block structures that can be homopolymeric (poly[β-D-mannosyluronic acid] [MM] and poly[α-L-gulosyluronic acid] [GG]) or heteropolymeric, i.e., containing a random block (MG). It is known that W-lyase can degrade any blocks, MM, GG, and MG (Haug and others 1967). The results of Figure 2 and 3 indicate that G-lyase has the same substrate specificity in degrading alginates as W-lyase.

Reaction of Mf with GAO and WAO

Figure 4 shows changes in the subunit components of Mf during the conjugation with GAO and WAO. In protein staining, the myosin heavy chain became a broadband whose mobility decreased as the reaction time increased. Additionally, the broad myosin heavy chain bands were detected by carbohydrate staining. These results indicate that GAO and WAO were covalently attached to the myosin heavy chains in Mf. Changes in the SDS-PAGE patterns of other protein components in Mf were also observed in the reaction with GAO; namely, the broadening of the actin band and the disappearance of tropomyosin were observed with an increase in the reaction time, as in the case of WAO.

Changes in the lysine residues and the AO bound to Mf with the progress of the glycosylation were examined to compare the reactivity for Mf of GAO and WAO. As shown in Figure 5, 10% and 22% of the available lysine content in Mf was diminished at the reaction with GAO for 24 h and 120 h, respectively, and the same lysine loss was observed in the reaction with WAO. Furthermore, the amount of AO bound to Mf increased simultaneously with the decrease in the available lysine. After the reaction for 120 h, the AO attached to Mf reached 117 μg/mg for GAO and 111 μg/mg for WAO. The results of Figure 5 indicate that there is no difference in the reactivity for Mf between GAO and WAO.

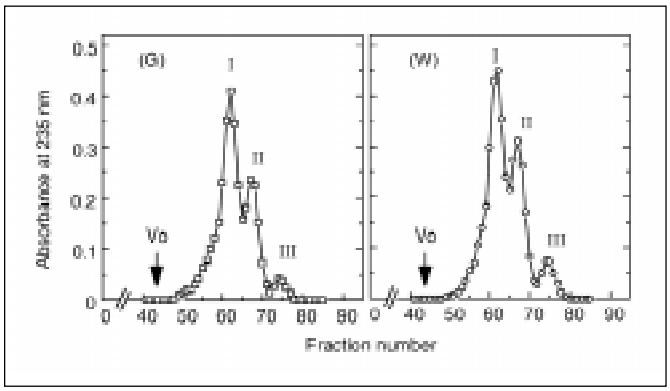

Figure 3—Gel-filtration pattern of GAO(G) and WAO (W). Vo shows void volume.

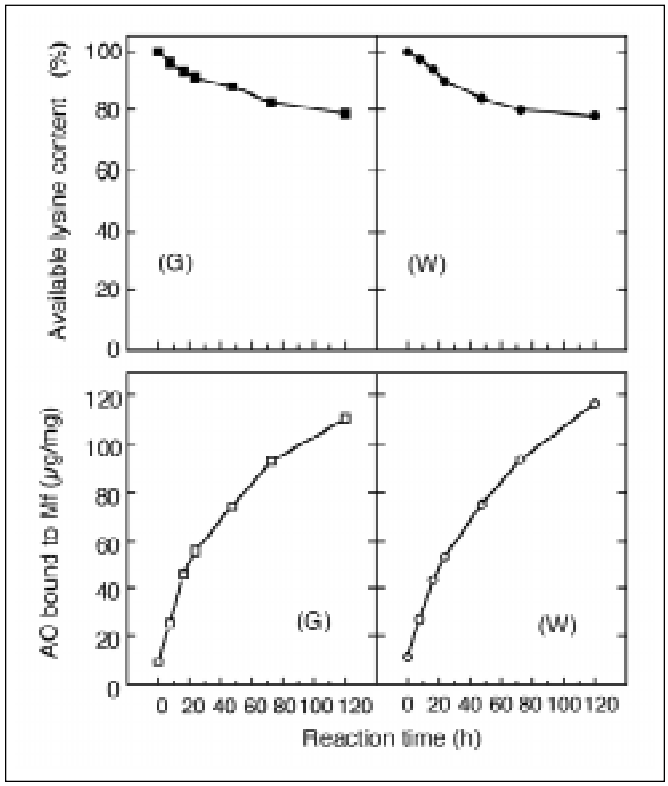

Figure 5—Changes in available lysine content and AO bound to Mf during conjugation with GAO (G) and WAO (W). Glycosylation was performed at 40 °C and 35% relative humidity.

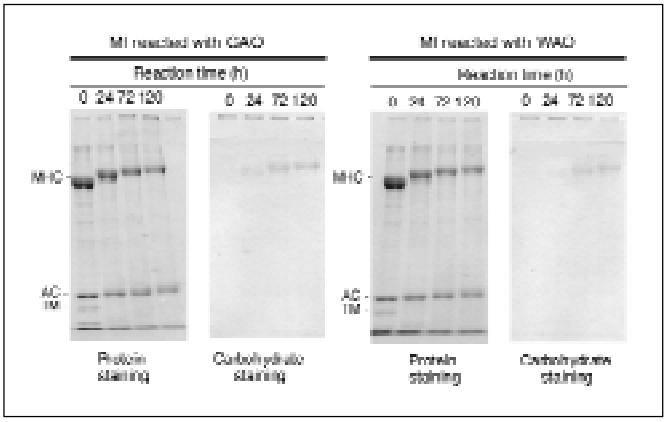

Figure 4—Change in SDS-PAGE pattern of Mf by conjugation with GAO and WAO. MHC, myosin heavy chain; AC, actin; TM, tropomyosin.

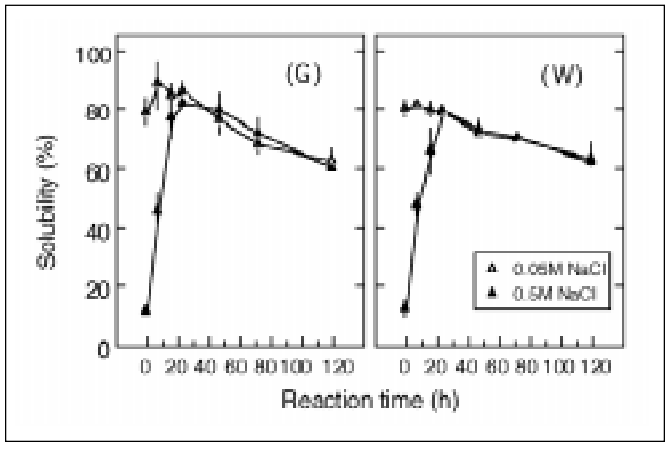

Figure 6—Changes in solubility of Mf in 0.05 M and 0.5 M NaCl during conjugation with GAO (G) and WAO (W). Reaction was performed at 40 °C and 35% relative humidity. Error bars represent standard errors of the means (*n* = 5).

Solubility and stability improvement of Mf by conjugation with AOs

Figure 6 shows changes in the solubility of Mf-AO conjugates in 0.05 M and 0.5 M NaCl (pH 7.5) with the progress of AO conjugation. After just lyophilization, the solubility levels of Mf-GAO and Mf-WAO in 0.5 M NaCl were 79% and 80%, respectively. Although the solubility slightly decreased with the progress of the AO conjugation, the solubility of Mf-GAO and Mf-WAO maintained at 62% at the reaction for 120 h. On the other hand, the solubility of Mf-GAO in 0.05 M NaCl increased markedly and reached the maximum value (81%) at the reaction for 24 h. Although the solubility in 0.05 M NaCl decreased slightly at the reaction for 24 to 120 h, this change was completely equivalent to the solubility change in 0.5 M NaCl. In addition, when the solubility of Mf in 0.05 M NaCl reached the maximum value, the amounts of GAO and WAO bound to Mf were 53 μg/mg and 56 μg/mg, respectively.

Table 1 shows the solubility loss of native Mf and Mf-AO conjugates by heating at 80 °C for 2 h. The solubility of native Mf in 0.5 M NaCl clearly decreased as a result of the heat treatment. On the contrary, the solubility of Mf-GAO containing 218 μg/mg of GAO hardly changed during the heat-treatment, regardless of the NaCl concentration. Additionally, there was no difference between Mf-GAO and Mf-WAO regarding the solubility level after the heat treatment. The results of Figure 6 and Table 1 indicate that the solubility in low-ionic-strength media and the thermal stability of Mf can be improved by conjugation with GAO, as in the case of WAO.

Table 1—Thermal stability of Mf-AO conjugate dissolved in 0.05 and 0.5 M NaCl.

Sample	Native Mf	Mf-GAO	Mf-WAO
AO bound to protein (μg/mg)	0	218	227
0.05 M NaCl (pH 7.5)	—	$97.4 \pm 2.8_b$	$97.0 \pm 2.2_b$
0.5 M NaCl (pH 7.5)	$42.4 \pm 6.3_a$	$94.9 \pm 3.7_b$	$95.1 \pm 4.9_b$

Mf-AO conjugates were prepared at 50 °C and 35% relative humidity for 120 h, and they were heated at 80 °C for 2 h. Different letters show significant difference ($P < 0.05$).

Conclusions

We confirmed that the production efficiency of AO increased with the introduction of genetic recombinant technology and that GAO has the ability to improve the solubility and the thermal stability of Mf. GAO seems to be an excellent material to prepare highly functional fish neoglycoprotein.

Acknowledgments

This study was partially supported by Regional New Consortium Project, the Ministry of Economy, Trade and Industry, Japan.

References

Aoki T, Kitahata K, Fukumoto T, Sugimoto Y, Ibrahim HR, Kimura T, Kato Y, Matsuda T. 1997. Improvement of functional properties of β-lactoglobulin by conjugation with glucose-6-phosphate through the Maillard reaction. Food Res Int 30:401–6.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–54.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–6.

Gacesa P. 1987. Alginate-modifying enzymes. A proposed unified mechanism of action for the lyases and epimerases. FEBS Lett 212:199–202.

Gornall AG, Bardwill CJ, David MM. 1949. Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751–66.

Haug A, Larsen B, Smidsrd O. 1967. Studies on the sequence of uronic acid residues in alginic acid. Acta Chem Scand 21:691–704.

Kato Y, Aoki T, Kato N, Nakamura R, Matsuda T. 1995. Modification of ovalbumin with glucose-6-phosphate by amino-carbonyl reaction. Improvement of protein heat stability and emulsifying activity. J Agric Food Chem 43:301–5.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–5.

Medina-Hernandez MJ, Guarcia-Alvarez-Coque MC. 1992. Available lysine in protein, assay using o-phthalaldehyde/*N*-acetyl-L-cysteine spectrophotometric method. J Food Sci 57:503–5.

Nakamura S, Ogawa M, Nakai S, Kato A, Kitts DD. 1998. Antioxidant activity of a Maillard-type phosvitin-galactomannan conjugate with emulsifying activity and heat stability. J Agric Food Chem 46:3958–63.

Nakamura S, Saito M, Goto T, Saeki H, Ogawa M, Gotoh M, Gohya Y, Hwang JK. 2000. Rapid formation of biologically active neoglycoprotein from lysozyme and xyloglucan hydrolysates through naturally occurring Maillard reaction. J Food Sci Nutr 5:65–9.

Old RW, Primrose SB. 1994. Expression in *E. coli* of cloned DNA molecules. In: Old RW, Primrose SB, editors. Principles of gene manipulation. 5th ed. London: Blackwell. p 139–66.

Saeki H. 1997. Preparation of neoglycoprotein from carp myofibrillar protein by Maillard reaction with glucose: Biochemical properties and emulsifying properties. J Agric Food Chem 45:680–4.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning, a laboratory manual. Vol. 1, 2nd ed. New York: Cold Spring Harbor Laboratory Press.

Sambrook J, Russell DW. 2001.Expression of cloned genes in *Escherichia coli*. In: Sambrook J, Russell DW, editors. Molecular cloning. 3rd ed. New York: Cold Spring Harbor Laboratory Press. p 15.1–15.65.

Sato R, Katayama S, Sawabe T, Saeki H. 2003. Stability and emulsion-forming ability of water-soluble fish myofibrillar protein prepared by conjugation with alginate oligosaccharide. J Agric Food Chem 51:4376–81.

Sato R, Sawabe T, Kishimura H, Hayashi K, Saeki H. 2000. Preparation of neoglycoprotein from carp myofibrillar protein and alginate oligosaccharide: Improved solubility in low ionic strength medium. J Agric Food Chem 48:17–22.

Sawabe T, Ezura Y, Kimura T. 1992. Purification and characterization of alginate lyase from marine *Altermonas* sp. Nippon Suisan Gakkaishi 58:521–7.

Sawabe T, Takahashi H, Ezura Y, Gacesa P. 2001. Cloning, sequence analysis and expression of *Pseudoalteromonas elyakovii* IAM 14594 gene (alyPEEC) encoding the extracellular alginate lyase. Carbohyd Res 335:11–21.

Somogyi M. 1952. Note on sugar determination. J Biol Chem 195:19–23.

Tsujino I, Saito T. 1962. Studies on alginate. A new unsaturated uronide insolated from hydrolysate. Agric Biol Chem 26:115–8.

Zacharius RM, Zell TE, Morrison JH, Woodlock JJ. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal Biochem 32:148–52.