Isolation and Characterization of Myosin from Walleye Pollack Surimi

Takao Ojima, Satoshi Yoshikawa, and Kiyoyoshi Nishita

Department of Marine Bioresources Chemistry, Faculty of Fisheries,
Hokkaido University, Hakodate, Hokkaido 041, Japan

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Actomyosin was extracted from myofibrils obtained from walleye pollack frozen surimi with 0.5 M KCl, 1 M sorbitol, and 1 mM Mg-ATP. Myosin was separated from F-actin by ultracentrifugation in the presence of MgCl₂ and ATP. The myosin consisted of heavy chains of Mr 200,000 and light chains of Mr 25,000, 18,000, and 17,000. Ca-ATPase activity of the myosin decreased approximately 20% per day at 0°C in 0.5 M KCl (pH 7.0) containing 1 M sorbitol. Ca-ATPase specific activity was approximately 8 times lower but EDTA-ATPase specific activity was several times higher than those of rabbit myosin at 0.05–0.5 M KCl, although the respective activities of both myosins showed similar dependences on KCl and pH. The Mg-ATPase activity of pollack myosin was increased approximately 100 times by the addition of an equal weight of rabbit F-actin and the activity was approximately 5 times higher than that of rabbit myosin. On the other hand, the actomyosin showed a significant increase in Mg-ATPase activity upon incubating at 20–30°C, probably due to some irreversible conformational changes of myosin. The pollack myosin formed mini-filaments of about 0.5 μ m length and the filaments tended to aggregate with each other.

Key words: walleye pollack, frozen surimi, myosin, ATPase, filament, morphology

Frozen surimi of walleye pollack is one of the most important materials used in the food processing industry especially for “kamaboko” in Japan and some foreign countries because of its high gel forming ability and mass productivity. To understand the relationship between the meat processing method and gel-forming mechanism, many investigations have been carried out using meat paste, myofibrils and myosin B of fish such as walleye pollack, carp, and some others^{1–6)} and warm-blooded vertebrates.^{7–9)} It has been shown that heat-induced gelation, which is closely related to the quality of the meat products, is dependent on the irreversible myosin polymerization in the myofibrils.^{7–11)} However, the molecular mechanism of the myosin polymerization is still obscure and detailed investigations are required in rather simple systems. Therefore, it is important to clarify the properties of the myosin from surimi.

Monomeric myosin of walleye pollack was isolated from surimi by Kimura *et al.*¹²⁾ using ammonium sulfate fractionation in the presence of 1 M sodium glutamate to prevent aggregation. However, its biochemical properties have not been investigated sufficiently.

In the present study, we describe the preparation of walleye pollack myosin from frozen surimi and its basic biochemical properties compared with those of rabbit myosin.

Materials and Methods

Frozen surimi (SA grade) of walleye pollack *Theragra chalcogramma* was kindly supplied by Nippon Suisan Co., Ltd. and stored at –40°C within one year. Rabbit myosin

and actin were prepared from the fast skeletal muscle by the methods of Perry¹³⁾ and Spudich and Watt,¹⁴⁾ respectively. Sorbitol was obtained from Towa Kasei Co., Ltd. and other reagents were of biochemical research grade.

ATPase activity was measured at 15°C in the presence of 5 mM CaCl₂ (Ca-ATPase), 5 mM EDTA (EDTA-ATPase), or 2 mM MgCl₂ (Mg-ATPase), in 0.05–0.5 M KCl, 25 mM Tris maleate (pH 7.0), 1 mM ATP, and 0.05 mg/ml myosin as follows: the pollack myosin (0.5 ml) was added to the ice-cold reaction mixture (4 ml) and preincubated at 15°C for 5 min, then 0.5 ml of 10 mM ATP was added to initiate ATPase reaction. At appropriate intervals (1–6 min), 1 ml of the reaction mixture was pipetted out and added to 2 ml of 15% trichloroacetic acid to terminate the reaction, then liberated inorganic phosphate was determined by the method of Youngburg and Youngburg.¹⁵⁾ Actin-activated Mg-ATPase activity was measured in the presence of 0.01–0.1 mg/ml F-actin and 50 mM KCl. SDS-PAGE was carried out by the method of Porzio and Pearson.¹⁶⁾ The protein concentration was determined by the biuret method¹⁷⁾ using bovine serum albumin Fraction V as a standard protein.

Walleye pollack myosin was prepared as follows: frozen surimi (6.5 g) was homogenized with 65 ml of 1 M sorbitol, 50 mM KCl, 20 mM Tris maleate (pH 7.0), and 5 mM 2-mercaptoethanol, in a Waring blender for 15 s; homogenization was repeated four times. The homogenate was then mixed with 65 ml of the same solution and centrifuged at 10,000 $\times g$ for 10 min to remove the supernatant. The washing was repeated twice more. The resulting precipitates of myofibrils were then suspended with 65 ml of 1 mM sodium bicarbonate and 5 mM 2-mercaptoethanol to remove

troponin-tropomyosin* from the myofibrils. After gentle stirring for 10 min at 0°C, the suspension was centrifuged at $10,000 \times g$ for 10 min to collect the precipitates. This washing procedure was repeated once more since it was essential to minimize the contamination of tropomyosin-troponin, which were hardly removed from the myosin by further purification. Then, the myofibrils were mixed with 65 ml of 0.5 M KCl, 1 M sorbitol, 20 mM Tris maleate (pH 7.0), 1 mM $MgCl_2$, 1 mM ATP, and 5 mM 2-mercaptoethanol to extract actomyosin. After 15 min, the mixture was centrifuged at $10,000 \times g$ for 15 min, and the supernatant obtained was added to 650 ml of cold distilled water. The precipitated actomyosin was collected by centrifugation at $10,000 \times g$ for 15 min, and dissolved in 1 M sorbitol, 0.5 M KCl, and 20 mM Tris maleate (pH 7.0). Actomyosin was then dissociated to actin and myosin by the addition of 0.2 M $MgCl_2$ and 5 mM ATP, and actin was removed by ultracentrifugation at $100,000 \times g$ for 1 h. The supernatant was diluted with 10 volumes of cold distilled water containing 5 mM 2-mercaptoethanol, and precipitated myosin was collected by centrifugation at $10,000 \times g$ for 10 min. The myosin was dissolved in and dialyzed against 1 M sorbitol, 0.5 M KCl, 50 mM Tris maleate (pH 7.0), and 5 mM 2-mercaptoethanol, and then ultracentrifuged at $100,000 \times g$ for 1 h before use. The yield was 100–120 mg myosin from 6.5 g of surimi.

Myosin filaments were formed as follows: 0.5 ml of myosin solution (1.0 mg/ml) in 1 M sorbitol, 0.5 M KCl, 50 mM Tris maleate (pH 7.0), and 5 mM 2-mercaptoethanol was transferred into a test tube of 1.7 cm diameter. It was then diluted slowly by adding 4 vol of 20 mM Tris maleate (pH 7.0) under continuous stirring with a magnetic stirrer, so as to make the final concentration of 0.2 mg/ml myosin, 0.2 M sorbitol, 0.1 M KCl, 20 mM Tris maleate (pH 7.0), and 1 mM 2-mercaptoethanol taking 1.5 min. After incubation at 8°C for 5 min, the filaments formed were applied to a grid, negatively stained with 2% uranyl acetate, and then observed by a Hitachi H-7000 electron microscope at an acceleration voltage of 75 kV.

Results and Discussion

Preparation of Myosin

Myosin was prepared by the method described in "Materials and Methods" which is the same method as used for scallop myosin¹⁸ except for the use of 1 M sorbitol to prevent protein denaturation.

Judging from SDS-PAGE (Fig. 1), the pollack myosin was contaminated by less than two percent of actin and was practically free from tropomyosin-troponin, and consisted of heavy chain of Mr 200,000, and light chains of Mr 25,000, 18,000, and 17,000 similarly to carp and tilapia myosins.¹⁹

ATPase Activity of Pollack Myosin

Changes in Ca-ATPase activity of the pollack myosin were examined during storage in 1 M sorbitol, 0.5 M KCl, 50 mM Tris maleate (pH 7.0), and 5 mM 2-mercaptoethanol at 0°C. As shown in Fig. 2, the activity

Fig. 1. SDS-polyacrylamide gel electrophoresis of the walleye pollack myosin.

Electrophoresis was carried out by the method of Porzio and Pearson¹⁶ with 10% polyacrylamide and 0.1% SDS. Coomassie Brilliant Blue R-250 was used to stain the gel. Pol. Mf, pollack myofibrils (15 μ g); Pol. M, pollack myosin (5 μ g); Rab. M, rabbit myosin (5 μ g). MHC, myosin heavy chain (Mr 200,000); A, actin (Mr 43,000); Tm, tropomyosin (Mr 34,000); TnT, troponin-T (Mr 29,000); LC1, myosin light chain-1 (Mr 25,000); TnC, Troponin-C (Mr 22,000); TnI, Troponin-I (Mr 21,000); LC2, myosin light chain-2 (Mr 18,000); LC3, myosin light chain-3 (Mr 17,000); A1-LC, myosin alkali light chain-1 (Mr 26,000); DTNB-LC, myosin DTNB-light chain (Mr 17,500); A2-LC, myosin alkali light chain-2 (Mr 15,500).

Fig. 2. Decrease in Ca-ATPase activity of the walleye pollack myosin during storage.

The pollack myosin (10 mg/ml) was stored at 0°C in 1 M sorbitol, 0.5 M KCl, 50 mM Tris maleate (pH 7.0), and 5 mM 2-mercaptoethanol. The storage time (day) of the myosin is indicated on the abscissa.

* According to our preliminary experiments, the proteins of Mr 34,000, 29,000, 22,000, and 21,000 have been assigned as tropomyosin, troponin-T, troponin-C, and troponin-I, respectively.

decreased about 20% per day, and its half life was about 2.5 days. Accordingly, we used a myosin preparation as soon as possible, at least within 2 days.

The KCl-dependences of the Ca- and EDTA-ATPase activities of the pollack and rabbit myosins were determined at 15°C as shown in Fig. 3. Comparing with rabbit myosin, pollack myosin showed approximately 8 times lower Ca-ATPase activity at 0.05–0.5 M KCl, while both myosins showed the same KCl-dependence. That is, the Ca-ATPase activities of both pollack and rabbit myosins simply decreased with increase in KCl concentration. On the other hand, the EDTA-ATPase activity of pollack myosin also showed similar KCl-dependence to that of rabbit myosin, however, it was activated more readily with lower KCl concentration than that of rabbit myosin below 0.5 M KCl. Furthermore, the activity of pollack myosin decreased at KCl concentrations higher than 0.5 M, probably because of denaturation of the myosin by salt.

The pH-dependences of Ca- and EDTA-ATPase activities of the pollack and rabbit myosins are shown in Fig. 4A and B. The maximal Ca-ATPase activity of the pollack myosin was seen at pH 6.4 and 8.8, and the minimum at around pH 7.2–7.6, while the maximal EDTA-ATPase activity was seen at pH 8.0. The Ca-ATPase activity in the pH range higher than 8.8 and the EDTA-ATPase activity in the pH range higher than 8.0 decreased unlike the activities of rabbit myosin. The decreases may be partly due to denaturation at alkaline pH.

Figure 5 shows the Mg-ATPase activity of the pollack and rabbit myosins in the presence of various amounts of F-actin. The activity of pollack myosin increased approximately 100 times by the addition of an equal weight of rabbit F-actin, and the specific activity was approximately 5 times higher than that of rabbit myosin at a weight ratio of 1:1 for myosin:F-actin. This strong actin-activation for pollack myosin Mg-ATPase was also observed in myosin prepared from fresh muscle of pollack (data not shown).

Fig. 3. Dependence on KCl concentration of Ca- and EDTA-ATPase activities of the walleye pollack and rabbit myosins.

ATPase activity was measured at 15°C in a following medium: A, 5 mM CaCl_2 , 25 mM Tris maleate (pH 7.0), 1 mM ATP, 0.05 mg/ml myosin, 0.1 M sorbitol, and various concentrations of KCl; B, 5 mM EDTA, 25 mM Tris maleate (pH 7.0), 1 mM ATP, 0.05 mg/ml myosin, 0.1 M sorbitol, and various concentrations of KCl. $\circ$, pollack myosin; $\bullet$, rabbit myosin.

Fig. 4. Dependence on pH of Ca- and EDTA-ATPase activities of the walleye pollack and rabbit myosins.

ATPase activity was measured at 15°C in the following medium: A, 0.5 M KCl, 5 mM CaCl_2 , 0.1 M sorbitol, 0.05 mg/ml myosin, 1 mM ATP, 50 mM Tris-HCl (pH 7–10) or Tris maleate (pH 4–7); B, 0.5 M KCl, 5 mM EDTA, 0.1 M sorbitol, 0.05 mg/ml myosin, 1 mM ATP, 50 mM Tris-HCl (pH 7–10) or Tris maleate (pH 4–7). The pH value indicated in the figures was the value of the medium measured immediately after ATPase assay. $\circ$, pollack myosin; $\bullet$, rabbit myosin.

Fig. 5. Mg-ATPase activity of the walleye pollack myosin in the presence of various amounts of F-actin.

The pollack myosin was combined with rabbit F-actin in various weight ratios in 1 M sorbitol, 0.5 M KCl, 50 mM Tris maleate (pH 7.0), and 5 mM 2-mercaptoethanol. Mg-ATPase activity was measured at 15°C after preincubation for 5 min at the same temperature in a medium containing 50 mM KCl, 2 mM MgCl_2 , 25 mM Tris maleate (pH 7.0), 0.1 M sorbitol, 1 mM ATP, 0.05 mg/ml myosin and various concentrations of rabbit F-actin. $\circ$, pollack myosin; $\bullet$, rabbit myosin.

Since these features seem to be characteristic to pollack myosin, we are now investigating the physiological significance of the strong actin-activation.

On the other hand, Taguchi *et al.* reported that walleye pollack myosin B showed heat-induced activation of Mg-ATPase activity.²⁰ Therefore, we undertook a similar experiment using actomyosin reconstituted from the pollack

Fig. 6. Heat-induced activation of Mg-ATPase activity of acto-walleye pollack myosin.

The pollack myosin was combined with rabbit F-actin at a weight ratio of 1:1 in 1 M sorbitol, 0.5 M KCl, 50 mM Tris maleate (pH 7.0), and 5 mM 2-mercaptoethanol. Then, the actomyosin was preincubated either for 0–60 min at 15°C in the Mg-ATPase assay medium (A), or for 5 min at indicated temperature on the abscissa and immediately cooled in a ice-bath (B). Then, Mg-ATPase activity was measured at 15°C after preincubation at the same temperature for 5 min.

Fig. 7. Turbidity of the walleye pollack myosin as a function of KCl concentration.

The pollack and rabbit myosins were incubated at 8°C (○) and 25°C (△, ●) for 5 min in a medium containing 25 mM Tris maleate (pH 7.0), 0.1 M sorbitol, 0.2 mg/ml myosin, and various concentrations of KCl. Turbidity of the myosin suspension was determined by measuring the absorbance at 350 nm. ○ and △, pollack myosin; ●, rabbit myosin.

myosin and rabbit F-actin. At first, we measured the Mg-ATPase activity of the actomyosin at 15°C immediately after preincubation for various times at the same temperature in a reaction mixture. As a result, the activity was gradually increased as the preincubation time was elongated (Fig. 6A). Subsequently, the actomyosin was incubated at 10–30°C for 5 min and immediately cooled in an ice-water bath, and then the Mg-ATPase activity was measured as above. As seen in Fig. 6B, the activity was sig-

Fig. 8. Electron micrographs of thick filaments of the walleye pollack myosin.

The myosin filaments prepared were applied to a grid, negatively stained with 2% uranyl acetate, and then observed with a Hitachi H-7000 electron microscope as described in the text. A and B, pollack myosin; C and D, rabbit myosin. Calibration bar in the photograph indicates 0.5 μm.

nificantly increased as the incubation temperature was raised. Thus, the heat-induced activation of the Mg-ATPase activity was also observed in the reconstituted actomyosin, irrespective of the species of actin. Therefore, such a high activation appeared to be attributable to heat-induced conformational changes of the pollack myosin.

Filament Formation of Pollack Myosin

It has been reported that some fish myosins form short thick filaments, so-called mini-filaments.^{21,22} We therefore examined the filament forming ability of the pollack myosin by turbidity measurement and electron microscopic observation. As shown in Fig. 7, the pollack myosin exhibited significantly higher turbidity than rabbit myosin at 0.05–0.15 M KCl. Even at 0.1–0.12 M KCl where rabbit myosin shows low turbidity and forms fairly homogeneous thick filaments (see Fig. 8), the pollack myosin showed high turbidity at 8°C and the maximum turbidity at 25°C. Then, we observed the morphology of pollack myosin filaments by electron microscope (Fig. 8A–D). At a higher magnification ($\times 30,000$, Fig. 8B), the bipolar filaments of about 0.5 μm in length were observed. Thus, the pollack myosin appeared to form mini-filaments like some other fish myosins.^{20,21} Moreover, the mini-filaments were found to gather on a grid at a lower magnification ($\times 10,000$, Fig. 8A).

To understand the gel forming mechanism of the walleye pollack surimi, more detailed studies are required on the properties and structure of the myosin.

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