Stability of Sardine Myofibrils during Storage at Various pH Values

Mohamed Kamal,*¹ Shugo Watabe,*¹ and Kanehisa Hashimoto*^{1,2}

(Received August 10, 1989)

The effect of pH on the denaturation profile of sardine ordinary and dark muscle myofibrils under various storage conditions was investigated by measuring Mg^{2+} -ATPase activity and solubility. Storage at 0°C for 2 days gave rise to two activity maxima both in the presence and absence of Ca^{2+} , one at pH around 7.5 and another at pH 9.5, and the highest Ca-sensitivity at pH 7.5 regardless of muscle type. After 2 days of storage at -20°C, the remaining activities were maximal at pH around 9.5 either in the presence or absence of Ca^{2+} , while extremely low at pH between 5.5 and 7.5 for both myofibrils. When myofibrils were incubated at 35°C for 30 min, Mg^{2+} -ATPase activities in the presence and absence of Ca^{2+} were both increased with the maximum values at pH 7 for ordinary muscle and at pH 7.5 for dark muscle myofibrils. Ca-sensitivity of ordinary muscle myofibrils almost disappeared while that of dark muscle to some extent remained, especially in alkaline pH range.

There was no marked change in solubility and SDS-PAGE pattern of soluble protein fraction throughout pH examined with both myofibrils stored either at 0 or -20°C. The solubility of ordinary muscle myofibrils decreased below 40% at any pH after incubation at 35°C whereas SDS-PAGE pattern showed no myosin heavy chain in the soluble protein fraction. The solubility of dark muscle myofibrils also decreased considerably, especially in an alkaline pH range. This well corresponded to the SDS-PAGE pattern of the soluble protein fraction where less amount of myosin heavy chain was observed at pH above 7.5.

The largest in quantity among caught in Japan is the sardine *Sardinops melanosticta*. The utilization of this fish as human food is, however, very low. One of the problems facing the effective utilization of sardine may be associated with a rapid loss of protein quality during storage. For improvement of the method to store this unstable fish meat, deeper knowledge seems necessary on the mechanisms underlying denaturation of the muscle proteins. For this purpose, some properties such as composition and thermal denaturation have been investigated on the sardine ordinary and dark muscle proteins.^{1,2)} Myofibril, one of the main muscle components has an enzymatic activity to split ATP. Therefore, by measuring this activity during storage, it was hoped to provide a measure of denaturation. In the previous study, we reported the changes in Ca^{2+} -ATPase activity of the sardine ordinary and dark muscle myofibrils during storage at various pH values and disclosed a significant correlation between storage conditions and denaturation of myofibrillar proteins.³⁾ To evaluate more precisely on the

quality of sardine myofibrillar proteins, it seems important to investigate the possible changes in Mg^{2+} -ATPase activity under similar storage conditions where Ca^{2+} -ATPase activity was followed, since the contribution of Ca^{2+} and Mg^{2+} to the mechanism of ATP hydrolysis is different.^{4,5)} In addition, Mg^{2+} -ATPase activity is regulated by small changes in a low concentration of Ca^{2+} through regulatory proteins such as troponin and tropomyosin.⁶⁾ In association with these strategies, Seki *et al.*⁷⁾ reported that Mg^{2+} -ATPase activity in the presence of Ca^{2+} of carp ordinary myofibrils increased during 30 days of ice storage at pH 8.4 as did Ca^{2+} -ATPase activity, while the activity in the absence of Ca^{2+} markedly increased in pH ranges including 5.6, 6.1 and 8.4. Seki *et al.*⁸⁾ also reported that sardine ordinary myofibrillar Ca^{2+} -, EDTA-, and Mg^{2+} -ATPase activities both in the presence and absence of Ca^{2+} decreased considerably during 10 days of ice storage when isolated myofibrils were stored at pH 6, whereas little change occurred at pH 7. As for frozen storage, a number of papers have been

*¹ Laboratory of Marine Biochemistry, Faculty of Agriculture, The University of Tokyo, Bunkyo, Tokyo 113, Japan (Md. Kamal, 渡部終五, 橋本周久: 東京大学農学部水産化学研究室).

*² Present address: Food Science Laboratory, Faculty of Education, Ibaraki University, Mito, Ibaraki 310, Japan (現所属: 茨城大学教育学部食物学研究室).

published on fish myosin, actomyosin and myofibrils dealing with changes in reduced viscosity,⁹⁾ active SH groups,¹⁰⁾ and Ca^{2+} -ATPase activity.¹¹⁻¹³⁾ However very little is known on changes in Mg^{2+} -ATPase activity of fish myofibrillar proteins during frozen storage. Furthermore, sardine has a high content of superficial dark muscle, the properties of which are markedly different from those of the ordinary muscle.¹⁴⁾

In this situation, Mg^{2+} -ATPase activity of the sardine's ordinary and dark muscle myofibrils was monitored during various types of storage. Solubility changes were also followed for both myofibrils to assess the denaturation profile more clearly.

Materials and Methods

Materials

Live specimens of sardine *Sardinops melanosticta* (average body weight, 69 g) were captured off Misaki, Kanagawa Prefecture. Immediately after each catch, specimens were killed by decapitation and transported to the laboratory in ice. The ordinary and dark myotomal muscles were excised at 4°C, taking care to minimize cross-contamination of fiber types. The ordinary muscle was dissected from the dorsal part, while the dark muscle from the whole trunk under the lateral line. Some sardine specimens were stored at -80°C in polyethylene bags for preparation of myofibrils when needed.

Preparation of Myofibrils

Myofibrils were prepared from ordinary and dark muscles immediately after excision according to Perry and Grey¹⁵⁾ in the presence of 0.1 mM dithiothreitol (DTT) throughout as reported previously.³⁾ Well washed myofibrils were suspended in 39 mM borate buffer (pH 7.1) containing 0.1 M KCl and 0.1 mM DTT at a concentration of 10-15 mg/ml.

Storage Conditions of Myofibrils and Mg^{2+} -ATPase Assay

Myofibrils (5 mg/ml) were stored in 1 ml of 0.1 M KCl plus 10 mM Tris-maleate (pH 5.5-8.0) or glycine-KOH (pH 8.5-9.6). Myofibril samples thus prepared were stored at 0°C for 2 days, -20°C for 2 days, or incubated at 35°C for 30 min. After storage, Mg^{2+} -ATPase activity was assayed at 25°C as reported previously³⁾ in 40 mM Tris-maleate (pH 7.0) containing 5 mM MgCl_2 , 50 mM KCl, 1 mM ATP and 0.5 mg/ml of myofibril with

either 0.25 mM CaCl_2 or 1 mM ethylene glycol bis (β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) in a final volume of 10 ml. The pH of the reaction mixture was around 7 during ATPase assay. Inorganic phosphate liberated was analyzed by the method of Fiske and SubbaRow.¹⁶⁾

Solubility Test

Myofibrils suspension (5 mg/ml) in 2 ml of 0.1 M KCl plus 10 mM Tris-maleate (pH 5.5-8.0) or glycine-KOH (pH 8.5-9.4) was stored essentially under the same conditions as before. After storage, each tube was taken out, thawed or cooled at 4°C and homogenized with 2 ml of 1 M KCl plus 100 mM phosphate buffer (pH 7.0) using a Polytron PTA 7 K. The homogenate was centrifuged for 30 min at $900 \times g$ and 4°C and protein content in the supernatant was determined by the biuret method.¹⁷⁾

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli¹⁸⁾ using 7.5-20% gradient slab gels containing 0.1% SDS. A 100 μl of soluble protein fraction was mixed with 100 μl of distilled water, 200 μl of 0.125 M Tris-HCl (pH 6.8) containing 50% glycerol and 1% SDS, and 40 μl of 2-mercaptoethanol, then treated at 100°C for about one min. A 50 μl of the sample thus treated was loaded on the gel. After the run, gels were stained with Coomassie brilliant blue R-250.

Results

Myofibrillar Mg^{2+} -ATPase Activity of the Ordinary and Dark Muscles

Mg^{2+} -ATPase activity was assayed in the presence of activating (0.25 mM CaCl_2) and relaxing (1 mM EGTA) buffers after storage at various temperatures and pH values. After ice storage, the activity curve of the sardine ordinary muscle myofibrils in the presence of Ca^{2+} showed a relatively sharp peak at pH around 9 and a small dull one at around 7.5 with activities of 0.45 and 0.42 $\mu\text{mol Pi/min}\cdot\text{mg}$, respectively (Fig. 1A). ATPase activity measured in the absence of Ca^{2+} similarly showed two maxima, one at pH around 6.5 (0.18 $\mu\text{mol Pi/min}\cdot\text{mg}$) and another around 9 (0.19 $\mu\text{mol Pi/min}\cdot\text{mg}$). The activity was lowest at pH 5.5 in both buffers as far as the present pH range is concerned. The maximum Ca-sensitivity of 67% was observed at pH around 7.5. In order to determine whether ice storage

Fig. 1. Influence of pH on Mg^{2+} -ATPase activity and Ca-sensitivity of sardine ordinary (A) and dark (B) muscle myofibrils after storage at 0°C for 2 days. Mg^{2+} -ATPase activity of ordinary (A) and dark (B) muscle myofibrils was measured in the presence of 0.25 mM $CaCl_2$ (□, ■; I) and 1 mM EGTA (○, ●; II) and Ca-sensitivity was calculated as $(I-II) \times 100 / I$ (△, ▲).

has any effect on the changes in Mg^{2+} -ATPase activity, a preliminary experiment was conducted by storing sardine ordinary myofibrils at 0°C and pH 7. A very little decrease in activity was evident after 2 days of storage in Ca^{2+} -activating buffer while EGTA-modified Mg^{2+} -ATPase activity remained essentially unchanged (data not shown). Ca-sensitivity was 70–75% immediately after preparation of myofibrils at pH 7.

After ice storage for 2 days, dark muscle myofibrils showed a pH-remaining activity pattern almost similar to that of ordinary muscle myofibrils (Fig. 1B). Unlike ordinary muscle myofibrils, dark

muscle myofibrils showed Mg^{2+} -ATPase activities both in the presence and absence of Ca^{2+} comparatively stable in the acidic pH region. The maximum value was observed at pH 9.3 both in activating and relaxing buffers with 0.32 and 0.17 $\mu\text{mol Pi/min}\cdot\text{mg}$, respectively. The highest remaining Ca-sensitivity was 53% at pH 7.5. Mg^{2+} -ATPase activities in the presence and absence of Ca^{2+} were 0.28 and 0.13 $\mu\text{mol Pi/min}\cdot\text{mg}$, respectively, immediately after preparation at pH 7, resulting in Ca-sensitivity of 54%.

Frozen storage resulted in aggregation of ordinary muscle myofibrils at any pH. When myo-

Fig. 2. Influence of pH on Mg^{2+} -ATPase activity and Ca-sensitivity of sardine ordinary (A) and dark (B) muscle myofibrils after storage at $-20^{\circ}C$ for 2 days. Refer to the legend in Fig. 1 for symbols and further details.

fibrillar Mg^{2+} -ATPase activity was assayed, the remaining maximum was obtained at pH 9.3 in both activating and relaxing buffers with 0.36 and 0.21 $\mu\text{mol Pi/min}\cdot\text{mg}$, respectively, although it was hardly observed at pH lower than 7.5 (Fig. 2A). Ca-sensitivity was relatively high at an alkaline pH with the maximum of 43% at pH 9.3.

Dark muscle myofibrils aggregated after frozen storage. When they were assayed for Mg^{2+} -ATPase activity, the remaining maximum was observed again at pH 9.3 both in the presence and absence of Ca^{2+} with 0.13 and 0.09 $\mu\text{mol Pi/min}\cdot\text{mg}$, respectively (Fig. 2B). The pH-remaining activity pattern was quite similar to that observed for ordinary muscle myofibrils (Fig. 2A), although the specific activity of dark muscle myofibrils was much lower. Ca-sensitivity maximum was 35%

at pH 9.

When sardine ordinary myofibrils were incubated at $35^{\circ}C$ for 30 min, Mg^{2+} -ATPase activities in the presence and absence of Ca^{2+} were markedly increased and their maxima were observed both at around pH 7 with 0.70 and 0.63 $\mu\text{mol Pi/min}\cdot\text{mg}$, respectively, as shown in Fig. 3A (see the specific activity after ice storage at neutral pH, Fig. 1A, as a control). The curve gradually declined with a subsequent rise of pH over 7 and rapidly above pH 9. Both activities decreased rapidly at pH below 6.5 and almost disappeared at pH 5.5. On the other hand, Ca-sensitivity almost disappeared irrespective of pH, after preparation of myofibrils at pH 7 (data not shown).

When dark muscle myofibrils were incubated at $35^{\circ}C$, Mg^{2+} -ATPase in activating and relaxing

Fig. 3. Influence of pH on Mg^{2+} -ATPase activity and Ca-sensitivity of sardine myofibrils after incubation at 35°C for 30 min. Refer to the legend in Fig. 1 for symbols and further details.

buffers showed the activity maximum both at pH around 7.5 with 0.46 and 0.32 $\mu\text{mol Pi/min}\cdot\text{mg}$, respectively (Fig. 3B). Unlike ordinary muscle myofibrils, dark muscle counterparts showed great decrease in activity at alkaline pH. Ca-sensitivity was considerably lower at any pH, but was less affected than that of ordinary muscle myofibrils, particularly in the pH range between 7.5 and 9.

Solubility and SDS-PAGE of Soluble Protein Fraction

To examine the effect of pH on the solubility of

sardine myofibrillar proteins, storage experiments were carried out at various pHs ranging from 5.8 to 9.4. Fig. 4 shows changes in solubility and SDS-PAGE pattern of soluble protein fraction after ice storage for 2 days. The solubility of dark muscle myofibrils was considerably high at any pH compared to that of ordinary muscle myofibrils. The maximum value was 84% with ordinary muscle myofibrils at pH 7.0 while 95% at pH around 7.5 with dark muscle myofibrils. There was no marked change in electrophoretic pattern for both ordinary and dark myofibrils throughout pH examined.

Fig. 4. Influence of pH on the solubility and SDS-PAGE pattern of soluble protein fraction of sardine myofibrils after storage at 0°C for 2 days. Sardine ordinary (□) and dark (■) muscle myofibrils were solubilized in 0.5 M KCl plus 50 mM phosphate buffer (pH 7), then soluble protein fraction of ordinary (A) and dark (B) muscle myofibrils were subjected to SDS-PAGE (top photographs).

As shown in Fig. 5, both sardine ordinary and dark muscle myofibrils showed the maximum solubility at pH 7.7 with 79% and 83%, respectively, after 2 days of frozen storage at -20°C. There was no apparent change in electrophoretic pattern of soluble protein fractions from ordinary and dark muscle myofibrils at any pH.

When incubated at 35°C for 30 min, ordinary myofibrillar proteins decreased in solubility below 40% irrespective of pH as shown in Fig. 6. Their solubility was higher than 85% at pH 7 immediately after preparation. In dark muscle myofibrils, the pattern of changes in solubility was varied depending on pH. The solubility was markedly

high in the acidic pH region with the maximum of 68% at pH 6.7, though still much lower than 98% of the control immediately after preparation. It was decreased with a subsequent rise of pH.

The top photographs in Fig. 6 show the changes in electrophoretic patterns of the soluble fractions after incubation at 35°C for 30 min. The ordinary muscle soluble protein fraction exhibited hardly any myosin heavy chain even after such a mild heat treatment. Actin band also became faint. The main protein band was probably that of troponin which was dissociated from aggregated actomyosin. On the other hand, myosin heavy chain was clearly observed in the soluble protein

Fig. 5. Influence of pH on the solubility and SDS-PAGE pattern of soluble protein fraction of sardine ordinary (A, △) and dark (B, ▲) muscle myofibrils after storage at -20°C for 2 days. Refer to the legend in Fig. 4 for further details.

fraction of dark muscle myofibrils, especially in the acidic pH region.

Discussion

Changes in Mg^{2+} -ATPase activity and solubility of sardine ordinary and dark muscle myofibrils during storage at various pH values have well been demonstrated. At 0°C , both sardine myofibrils exhibited the maximum remaining ATPase activity at alkaline pH around 9 both in the presence and absence of Ca^{2+} . Evidence has been presented elsewhere⁷⁾ that changes in ATPase activity and loss of Ca-sensitivity of myofibrils during ice storage, especially in an alkaline medium might be due to modification of actin-myosin interaction by the oxidation of the thiol groups of myosin moiety.

After frozen storage, both myofibrils showed the maximum remaining activity at alkaline pH, the activity being hardly seen at acidic and neutral pH in any Mg^{2+} -ATPase assay system. Unfortunately, the present phenomena were not clearly understood, thus any discussion on it must be at best speculative. It should be, however, emphasized that the hydrogen bond probably increased during frozen storage, and an interaction between hydrophobic and hydrogen bonds resulted in aggregation of myofibrillar proteins as found by Buttkus¹⁰⁾ with rabbit and trout myosins. This might be attributable to structural changes in myofibrillar proteins which would result in a decreased affinity between actin and myosin as observed by Ohnishi *et al.*¹⁰⁾ with carp ordinary muscle actomyosin during frozen storage. As a result of conformational changes, the interaction

Fig. 6. Influence of pH on the solubility and SDS-PAGE pattern of soluble protein fraction of sardine ordinary (A, ○) and dark (B, ●) muscle myofibrils after incubation at 35°C for 30 min. Refer to the legend in Fig. 4 for further details.

of myosin with actin might be reduced. It is therefore likely that the decreased activity in both Mg^{2+} assay systems at acidic and neutral pH with ordinary and dark muscle myofibrils was due to an increased proportion of ATPase activity catalyzed by myosin alone. However, a comparatively high ATPase activity at an alkaline pH might be argued that polymerization reaction of proteins is labile and partially reversible where dissociation readily occurs at moderately high pH due to the net negative charge of macromolecules.²⁰⁾

The increase of Mg^{2+} -ATPase activity and loss of Ca -sensitivity with both myofibrils either in the presence or absence of Ca^{2+} after a mild heat treatment could be due to conformational changes occurring in regulatory proteins of troponin-tropomyosin complex. It would seem then, at least in the case of sardine muscle, that the Ca^{2+} -

regulatory system does not function well at temperatures above the physiological range as found by Fuchs *et al.*²¹⁾ at 30°C with frog actomyosin and 40–45°C with rabbit skeletal actomyosin. Taguchi *et al.*²²⁾ reported that the increase of Mg^{2+} -ATPase activity with flying fish actomyosin during 50 min incubation at 35°C and pH 7 could be due to interaction between myosin and actin during thermal treatment.

Four more general observations can be made concerning the changes in Mg^{2+} -ATPase activity during storage at various pH values. At first, the myofibrillar ATPase activity of ordinary muscle was quite higher than that of dark muscle. This agreed with those reported for other fishes.^{23–25)} Secondly, the changes in myofibrillar ATPase activity of both muscles at 35 and –20°C were prominent compared to those of ice storage.

Thirdly, the low activity at pH 5.5 in sardine ordinary muscle myofibrils revealed the direct function of pH. Finally, it should be noted that the most prominent differences exist in pH dependency between remaining Ca^{2+} -³⁾ and Mg^{2+} -ATPase activities for both sardine ordinary and dark myofibrils when stored at -20°C . The maximum remaining Ca^{2+} -ATPase activity was observed at a pH level around 7.5, while extremely low Mg^{2+} -ATPase activity in this pH region irrespective of muscle type. Therefore, it can be concluded that myofibrillar Mg^{2+} -ATPase activity is a sensitive parameter to know myofibrillar denaturation during frozen storage in the neutral pH region.

Unlike Mg^{2+} -ATPase activity, there was no marked change in solubility and electrophoretic pattern of the soluble fraction at any pH with both myofibrils stored either at 0 or -20°C . Although the solubility after ice and frozen storage was quite high throughout pH examined, it is probable that the solubility might decrease with the extended storage period as found by Seki *et al.*⁷⁾ with carp myofibrils during ice storage and Oguni *et al.*²⁶⁾ with carp actomyosin during frozen storage. The solubility in ordinary muscle myofibrils decreased to below 40% at any pH during heat treatment at 35°C for 30 min. The result was consistent with the SDS-PAGE pattern which showed no myosin heavy chain in soluble protein fraction throughout the pH range examined. Some types of aggregated actomyosin which is not salt-soluble would rather activate Mg^{2+} -ATPase activity. The solubility of dark muscle myofibrils also considerably decreased but remained much higher in weakly acidic and neutral pH ranges than in alkaline range. This well corresponded to SDS-PAGE patterns of the soluble protein fraction where less amount of myosin heavy chain was observed at pH above 7.5.

Acknowledgments

We are grateful to the staff of Kanagawa Prefectural Fisheries Experimental Station for their help in collecting sardine specimens.

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