Postmortem Changes in Myofibrillar ATPase of Sardine Ordinary and Dark Muscles

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Changes in myofibrillar ATPase activity of ordinary and dark muscle of sardine Sardinops melanosticta were examined in relation with rigor mortis progress and concomitant decline of muscle pH during 24 h postmortem period, along with changes during 10 days of ice and frozen storage.

Myofibrillar Ca²⁺- and EDTA-ATPase activities, and Mg²⁺-ATPase activity in the presence of Ca²⁺ of sardine ordinary muscle clearly decreased when “rigor index”, a parameter of rigor tension, proceeded to 50% and muscle pH fell to around 6.4. Mg²⁺-ATPase activity in the absence of Ca²⁺ showed a slight decrease under the same conditions. On the other hand, the ATPase activity of the dark muscle started decreasing only at full rigor when muscle pH fell to 6.4 in almost all assay systems, except Mg²⁺-ATPase activity in the absence of Ca²⁺ which hardly changed during rigor mortis progress. A marked decrease in the activity was observed with both sardine muscles during extended ice storage of 10 days.

Both sardine muscles showed a large fall in myofibrillar ATPase activity, when stored frozen for 10 days. With either muscle, pH decreased down to 5.8-5.9 after one day and remained essentially unchanged thereafter.

Sardine Sardinops melanosticta accounts for the largest part of recent fish catch in Japan. The usage of this fish as human consumption is however very low, probably due to a rapid loss of protein quality during storage. One of the reasons for deterioration of sardine muscle proteins is the quick decrease of muscle ATP concentration followed by a decline of muscle pH,¹⁻⁴ even after death while not struggling.⁴ Postmortem decline of sardine muscle pH markedly influences the quality of kamaboko gel¹,⁵ and myofibrillar solubility.⁶,⁷

For improving the method to store this unstable fish meat, deeper knowledge seems necessary on the mechanisms underlying denaturation of muscle proteins. For this purpose, we have recently examined the changes in ATPase activity and solubility of sardine muscle myofibrils during storage at various pH values,⁸,⁹ since myofibrillar ATPase activities are also associated with muscle protein quality.¹⁰ Ca²⁺-ATPase activity along with Mg²⁺-ATPase activity in the presence or absence of Ca²⁺, was extremely unstable in the acidic pH region, whereas the solubility remained high at any pH.

The underutilization of sardine stems also from limited information on the properties of dark muscle proteins which accounts for 20-30% of the total muscle.¹¹ This situation aroused us to examine changes in ATPase activities of sardine dark muscle myofibrils during storage at various pH values, since the dark muscle declines in pH as quickly as does the ordinary muscle.¹² Any ATPase activity of the dark muscle myofibril was to some extent more stable in the acid pH region than that of the ordinary muscle.⁸,¹⁰

Model experiments with isolated myofibrils as described above would not necessarily explain the changes occurring in intact muscle. Seki et al.¹¹ observed that isolated myofibrils of sardine ordinary muscle stored at pH 6 and 0°C declined both Ca²⁺-ATPase activity and Mg²⁺-ATPase activity in the presence or absence of Ca²⁺ more rapidly than activities of intact muscle stored at 0°C. However, they used as the starting materials sardine specimens which were 2-4 h after death.
It has been reported that the muscle pH of sardine declined quickly after death \(^1\) and even more when sardines were captured at the fishing ground and killed while struggling or after fatigue.\(^2\) Therefore, a possible effect of rigor mortis progress on muscle protein quality is not clearly known for sardines, although it is generally accepted that large changes in muscle ultrastructure proceed during the progress of rigor mortis.\(^3\) In other words, it is ambiguous whether sardine would deteriorate muscle quality in the very early stage after death. In addition, the effect of frozen storage on the quality of intact sardine muscle has not yet been demonstrated satisfactorily.

This paper deals with changes in myofibrillar ATPase activities of sardine in association with rigor mortis progress. The effect of frozen storage on sardine muscle quality was also examined using myofibrillar ATPase activities as parameters.

**Materials and Methods**

**Materials**

Live specimens of sardine (average body weight, 75 g) were captured off Misaki, Kanagawa Prefecture, maintained about one week in situ, and transported to the Kanagawa Prefectural Fisheries Experimental Station. Fish were held in tanks of 400 l which were supplied by running filtered, aerated seawater. They were fed daily for one month with a commercial fish meal. Sardine specimens were sacrificed by cutting the head immediately after taking from tanks with a small net and stored in ice. At selected time intervals, they were taken out from the storage container and measured for rigor index and muscle pH during the first 24 h storage. Myofibrils were prepared from ordinary and dark muscles of the same specimens during the first 24 h, and thereafter during 10 days of ice storage without measuring rigor index. In a separate series of experiments, live sardines (average body weight, 24 g) captured at the same location were killed by decapitation at the fishing ground immediately after catch, and transported to the laboratory in ice. They were then stored at 0, -20, and -80°C in polyethylene bags for 10 days and myofibrils were prepared at due time intervals.

**Rigor Index**

“Rigor index” of the fish was measured essentially according to Bito et al.\(^4\) and used as a parameter of rigor tension.\(^4\) Rigor indices of 0 and 100% represent pre-rigor and full-rigor states, respectively.

**pH**

Muscle pH was directly measured in intact muscle using a portable pH meter (Nisshinrika NPN-10S) with a pointed probe electrode.

**Preparation of Myofibril**

Two grams of ordinary and dark muscles were excised from each specimen, taking care to minimize cross contamination of fibre types. The ordinary muscle was dissected from the dorsal part, while the dark muscle from the whole trunk under the lateral line. Myofibrils were prepared essentially according to Perry and Grey.\(^5\) Briefly, the muscle was homogenized in 10 m/ of cold 39 mm borate (pH 7.1) containing 0.1 M KCl and 0.1 mm dithiothreitol (DTT), and centrifuged at 900 × g for 15 min. The residue was resuspended with the same buffer to remove the soluble sarcoplasmic proteins. The same procedure was repeated once more and residual myofibrils were resuspended at a concentration of 8-10 mg/ml in 39 mm borate (pH 7.1) containing 0.1 m KCl and 0.1 mm DTT.

**ATPase Assay**

ATPase activity was measured in a solution containing 0.2 mg/ml of myofibril, 1 mm ATP, 20 mm Tris-maleate (pH 7.5), and either 5 mm CaCl\(_2\) plus 0.1 m KCl for Ca\(^{2+}\)-ATPase or 2 mm EDTA plus 0.5 m KCl for EDTA-ATPase in a final volume of 10 ml. Mg\(^{2+}\)-ATPase activity was assayed in a solution containing 5 mm MgCl\(_2\), 1 mm ATP, 0.05 m KCl, 25 mm Tris-maleate (pH 7.5), 0.2 mg/ml of myofibril, and either 0.25 mm CaCl\(_2\) or 1 mm ethylene glycol bis (β-aminoethyl ether) N, N', N'-tetraacetic acid (EGTA) in a final volume of 10 ml. The reaction was started at 25°C by the addition of ATP to a preincubated medium containing myofibrils. Under continuous stirring, a 2 ml aliquot of reaction mixture was taken out at due time intervals and to it was added 1 m/ of precooled 15% trichloroacetic acid for termination of the reaction. Precipitated proteins were removed by centrifugation at 900 × g for 5 min, and an aliquot of clear supernatant was assayed for liberated inorganic phosphate by the method of Fiske and SubbaRow.\(^6\)

Ca\(^{2+}\)-sensitivity was calculated from Mg\(^{2+}\)-
ATPase activity in the presence of Ca\(^{2+}\) relative to that in the presence of EGTA as follows.

\[
\text{Ca}^{2+}-\text{sensitivity} = \frac{1}{2} \left( \frac{\text{EGTA-modified ATPase activity}}{\text{Ca}^{2+}-\text{modified ATPase activity}} \right) \times 100
\]

Results

Rigor Mortis Progress and Changes in Myofibrillar ATPase Activities

Rigor mortis progress of sardine in association with changes in pH during postmortem storage has been described in our previous paper. The onset of rigor mortis was 2 h after death. The rigor index slowly increased after 2-4 h followed by a rapid phase until the fish exhibited full rigor (100% rigor index) after 8 h. The rigor index decreased to some extent after 12-24 h, suggesting the occurrence of relaxation from rigor. Muscle pH values, 7.2 for sardine both ordinary and dark at the start, decreased rapidly until 6 h when they were 6.3 and 6.4, respectively. Muscle pH then declined and values after 24 h of storage ranged from 5.8 to 5.9 for the ordinary and from 5.9 to 6.0 for the dark muscle.

Myofibrillar ATPase activities of sardine ordinary muscle from the same specimens used for rigor index measurements, were assayed. The results were plotted against the rigor index from 0% for pre-rigor specimens to 100% for full-rigor ones 8 h after death (Fig. 1). Ca\(^{2+}\) and EDTA-ATPase activities and Mg\(^{2+}\)-ATPase activity in the presence of Ca\(^{2+}\) started decreasing.

![Graph A](image1)

![Graph B](image2)

Fig. 1. Relation of myofibrillar ATPase activities of sardine ordinary muscle with rigor index. A, Ca\(^{2+}\) (●) and EDTA-ATPase (○); B, Mg\(^{2+}\)-ATPase in the presence of 0.25 mm CaCl\(_2\) (□) or 1 mm EGTA (■), and Ca\(^{2+}\)-sensitivity (△).

![Graph A](image3)

![Graph B](image4)

Fig. 2. Relation of myofibrillar ATPase activities of sardine dark muscle with rigor index. A, Ca\(^{2+}\) (●) and EDTA-ATPase (○); B, Mg\(^{2+}\)-ATPase in the presence of 0.25 mm CaCl\(_2\) (□) or 1 mm EGTA (■), and Ca\(^{2+}\)-sensitivity (△).
when rigor index reached 50%, and further decreased at full rigor. Only a slight decrease in Mg\(^{2+}\)-ATPase activity in the absence of Ca\(^{2+}\) was, however, observed with the progress of rigor mortis, resulting in the decrease of Ca\(^{2+}\)-sensitivity from about 60% at death to around 50% at full rigor.

In the case of dark muscle, myofibrillar Ca\(^{2+}\)- and EDTA-ATPase activities decreased when sardine exhibited 100% rigor index (Fig. 2). Mg\(^{2+}\)-ATPase activity in the presence of Ca\(^{2+}\) decreased only slightly at full rigor, and the activity in the absence of Ca\(^{2+}\) hardly changed throughout. Therefore, Ca\(^{2+}\)-sensitivity remained almost constant at about 30%.

### Changes in Muscle pH and Myofibrillar ATPase Activities

Myofibrillar ATPase activities of the ordinary muscle were plotted against muscle pH of sardine immediately after death and those at various postmortem stages until 24 h when relaxation from rigor began (about 60% rigor index) (Fig. 3). Ca\(^{2+}\) - and EDTA-ATPase activities were unaffected in the range of muscle pH between 6.5 and 7.2, and decreased sharply when muscle pH fell below 6.4. Mg\(^{2+}\)-ATPase activity in the presence of Ca\(^{2+}\) also decreased markedly with decrease of muscle pH below 6.4, while the activity in the absence of Ca\(^{2+}\) did only slightly at pH around 6. Subsequently, Ca\(^{2+}\)-sensitivity of myofibrillar Mg\(^{2+}\)-ATPase of sardine ordinary muscle re-

![Fig. 3. Relation of myofibrillar ATPase activities of sardine ordinary muscle with muscle pH. A, Ca\(^{2+}\) - (○) and EDTA-ATPase (●); B, Mg\(^{2+}\)-ATPase in the presence of 0.25 mM CaCl\(_2\) (□) or 1 mM EGTA (■), and Ca\(^{2+}\)-sensitivity (△).](image)

![Fig. 4. Relation of myofibrillar ATPase activities of sardine dark muscle with muscle pH. A, Ca\(^{2+}\) - (○) and EDTA-ATPase (●); B, Mg\(^{2+}\)-ATPase in the presence of 0.25 mM CaCl\(_2\) (□) or 1 mM EGTA (■), and Ca\(^{2+}\)-sensitivity (△).](image)
mained unchanged at about 60% until the muscle pH fell below 6.4. Ca\(^{2+}\)-sensitivity decreased down to 50% or less as muscle pH declined below 6.

Figure 4 shows the relation of myofibrillar ATPase activities of the dark muscle with muscle pH. All ATPase activities began to decrease more or less when muscle pH declined to 6.4. However, Mg\(^{2+}\)-ATPase activity in the absence of Ca\(^{2+}\) decreased only slightly, resulting in the decrease of Ca\(^{2+}\)-sensitivity. Ratios of activities at around pH 6 to those at pH 7.2 of the dark muscle were 76, 65, 79, and 86% for Ca\(^{2+}\)-, EDTA-, Ca\(^{2+}\)-modified Mg\(^{2+}\)-, and EGTA-modified Mg\(^{2+}\)-ATPases, respectively. Since corresponding ratios for the ordinary muscle were 69, 52, 66, and 87% in the above order, myofibrillar ATPases of the dark muscle were to some extent more stable in the acidic pH region than those of the ordinary muscle, which agreed well with differences in ATPases of isolated myofibrils between both sardine muscles.8,9)

Changes in Myofibrillar ATPase Activities during Extended Ice Storage

Changes in myofibrillar ATPase activities of sardine ordinary and dark muscle during 10 days of storage at 0°C were examined. Ultimate pH ranged from 5.8 to 5.9 for both muscles. The results obtained with the ordinary muscle are shown in Fig. 5A. Different curves were obtained for respective ATPase activities, which all decreased continuously with the lapse of storage time. Ca\(^{2+}\)-ATPase activity decreased to about 55% of its initial level after 10 days of storage, while there was a large fall in EDTA-ATPase activity with loss of 90% after 10 days. Mg\(^{2+}\)-ATPase activity in the presence of Ca\(^{2+}\) decreased to its original during the same period, while the activity in the absence of Ca\(^{2+}\) was far less affected compared to those in other assay systems.

Changes in myofibrillar ATPase activity of sardine dark muscle during ice storage are shown in Fig. 5B. Ca\(^{2+}\)-ATPase activity decreased nearly in parallel with that of Mg\(^{2+}\)-ATPase activity in the absence of Ca\(^{2+}\), while EDTA-ATPase activity decreased in parallel with Mg\(^{2+}\)-ATPase activity in the presence of Ca\(^{2+}\). However, EDTA-ATPase activity remained about 30% of its original after 10 days of ice storage, the remaining ratio being higher than that of the ordinary muscle.

The Ca\(^{2+}\)-sensitivity of myofibrillar Mg\(^{2+}\)-ATPase of the ordinary muscle was 60% at the start, while only 30% with the dark muscle. It decreased to below 20 and 10% for ordinary and dark muscles, respectively, after 10 days of ice storage.

Changes in Myofibrillar ATPase Activities during Frozen Storage

At specified time intervals, ordinary and dark muscles were excised carefully from three sardines each during storage at 0, -20, and -80°C. Since sardines used in this series of experiment were small, muscle samples from three specimens were chopped and mixed together. The ultimate pH was again in a range from 5.8 to 5.9 for both
Fig. 6A shows changes in myofibrillar Ca\(^{2+}\)-ATPase activity of the ordinary muscle. Ca\(^{2+}\)-ATPase lost about one half of its original activity within 2 days regardless of storage temperature. The activity further decreased even at \(-20\) and \(-80^\circ\)C during the subsequent period of storage, although the decreasing rates were lower than that at \(0^\circ\)C. Ca\(^{2+}\)-ATPase activity remained at 30-40\% of its initial level after 10 days of storage in the order of \(-80<-20<-0^\circ\)C. Mg\(^{2+}\)-ATPase in the presence of Ca\(^{2+}\) also showed a large fall in the activity irrespective of storage temperature during the first 2 days, while the activity in the absence of Ca\(^{2+}\) was less affected (data not shown).

Figure 6B shows changes with the dark muscle. Ca\(^{2+}\)-ATPase activity decreased rapidly in all cases, losing about 40-50\% of its original within 2 days. At \(-20\) and \(-80^\circ\)C, the activity was more stable thereafter than at \(0^\circ\)C, as with the ordinary muscle (Fig. 6A). However, Mg\(^{2+}\)-ATPase activity in the absence of Ca\(^{2+}\) of the dark muscle decreased more rapidly than that of the ordinary muscle in contrast to the activity in the presence of Ca\(^{2+}\) which decreased similarly in both ordinary and dark muscles (data not shown).

**Discussion**

It is well known that pelagic fishes are very active in locomotion, leading to a high metabolic rate. Correspondingly, the postmortem changes of sardine are extremely rapid,\(^5\) compared to those of bottom fish reported elsewhere.\(^7\) Myofibrillar ATPase activities of sardine ordinary muscle clearly decreased when rigor index proceeded to 50\% and muscle pH fell below around 6.4. The activity of the dark muscle decreased only when sardines reached the full-rigor state, where pH around 6.4. Muscle pH continued to decrease after sardines reached the full-rigor state, attaining ultimate pH values from 5.8 to 5.9 for both muscles which led to a considerable decrease in the activity in all assay systems. Therefore, the decrease of myofibrillar ATPase activities seemed to be more closely related with changes in muscle pH than rigor mortis progress during the first 24 h postmortem storage of sardine. These results agreed with those reported for mackerel ordinary muscle\(^20\) for which during ice storage, myofibrillar Ca\(^{2+}\)-ATPase activity decreased after the fish attained the full-rigor state as muscle pH fell below 6. Therefore, the decrease in fish myofibrillar ATPase activities seemed to be a direct function of pH as for pig muscle.\(^21\) It has been suggested that the loss of myofibrillar ATPase activity under acidic pH conditions is due to denaturation, and not to proteolysis, of myosin.\(^22\) It is also worthwhile to note that myofibrillar ATPase activity of sardine ordinary muscle decreased more rapidly than that of the dark muscle in the acidic pH region. These results could be expected since dark muscle myosin and myofibrils were more stable in this pH region than those of the ordinary muscle counterparts.\(^8\) There was a large fall in myofibrillar ATPase activity with both sardine muscles during 10 days of ice storage (Fig. 5). Seki et al.\(^{15}\) found a
similar decrease in the activity with sardine ordinary muscle during 6 days of ice storage. In the present study, muscle pH decreased down to 5.8-5.9 within one day for both muscles and remained essentially unchanged thereafter. Therefore, the muscle maintained at a low pH for a long period seemed to reduce gradually ATPase activity as reported for rabbit muscle. Some lysosomal proteases which are active in the acidic pH region, might degrade muscle proteins during a longer term of storage, affecting myofibrillar ATPase activity.

The low Ca²⁺-sensitivity of myofibrillar Mg²⁺-ATPases of sardine ordinary and dark muscle even immediately after death was not explained. However, Seki et al. reported that it could be related with postmortem changes in sardine myofibrils. In contrast to their results, we did not observe any increase of Mg²⁺-ATPase activity in the absence of Ca²⁺ for both sardine muscles during ice storage.

A decrease in myofibrillar ATPase activity was also observed with both sardine muscles during frozen storage at -20 and -80°C. Fukuda et al. reported that sardine meat block stored frozen at -20°C for 8 months lost about one half of the initial Ca²⁺-ATPase activity. Mackerel muscle proteins also denatured much more rapidly when frozen at post-rigor where muscle pH was below 6, than when frozen at pre-rigor. Kelly demonstrated that the lower the cod fillet pH was, the more rapidly the muscle proteins deteriorated during frozen storage. In the present experiments of frozen storage, the ultimate pH for both sardine muscles ranged from 5.8 to 5.9. Therefore, a large fall in myofibrillar ATPase activity of sardine muscles during frozen storage was probably due to the potential denaturing effect of low pH. Side-to-side aggregation of myosin molecules has been claimed to occur as the major event in freeze-denaturation of fish muscle proteins.

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References


