Review

Myosin Denaturation Study for the Quality Evaluation of Fish Muscle-based Products

Kunihiko Konno*

Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido Japan 041-8611 Japan

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Myosin is the major protein in fish muscle and determines the quality of the muscle. Fish myosin is typically unstable, especially myosin from cold-water fish species. Accordingly, an understanding of species-specific myosin denaturation is important for evaluating the quality of fish muscle. The myosin molecule consists of a head and tail portion and thus the denaturation of these two regions was analyzed upon the heating and freezing of myofibrils (Mf). Moreover, preferential actin denaturation determines myosin denaturation in Mf upon treatment with high concentrations of salt or by freezing. The indices for detecting myosin and actin denaturation in Mf were successfully applied to evaluate the quality of Bluefin tuna samples and frozen surimi. In addition, the unique myosin denaturation observed with squid Mf is discussed.

Keywords: myosin, actin, denaturation, surimi, tuna, squid, myofibrils

1. Characteristic properties of fish myosin

1.1 Muscle structure Muscles that help fish swim are the major source of protein when fish are consumed as a protein resource. The fundamental contraction mechanism used by the fish dorsal muscle is the same as for mammalian skeletal muscle. The most abundant protein found in muscle tissue is myosin, which together with actin are the proteins responsible for muscle contraction. Myosin and actin are filamentous proteins in fish muscle. The regular arrangement of these two filaments in muscle fibers gives a regular striation pattern on the surface of the muscle (Huxley 1963). The smallest fibrous unit of muscle tissue for contraction is the myofibril (Mf). A single Mf fiber in muscle is very long and consists of repeated segments of the smallest contraction unit, the sarcomere. A phase-contrast microscope image of fish Mf is shown in Fig. 1. The regions connecting sarcomeres are called Z-lines. Myosin filaments (or thick filaments, depending on their thickness) located at the center of the sarcomere and F-actin filaments (or thin filaments) stretch from both sides of a Z-line and each occupy roughly 1/3rd the length of the sarcomere.

Homogenization of muscle often cleaves the Mf at the Z-line and produces fragmented fibers consisting of a small number (5 – 10) of sarcomeres (Fig. 1). It is this material that is generally termed ‘Mf’. This fragmented Mf is a convenient and useful material for studying myosin because Mf retains an intact arrangement of thick and thin filaments, as in muscle.

1.2 Basic functions of myosin and its structure Myosin has three important biological functions. A schematic model of a myosin molecule is shown in Fig. 2. The first function is the filament-forming ability of the myosin tail region (rod) at physiological ionic strength. The second function is to interact with its counterpart protein F-actin, and the third is its ATP hydrolytic ability to generate energy for contraction. The second and third functions are conducted by the head region (subfragment-1, S-1). The myosin molecule consists of two heavy chain components (HC, 200 kDa) and four light chain components with different properties (LC, ~20 kDa). The amino terminal half of a single myosin HC forms a globular structure. The carboxyl half adopts a mostly α-helical structure and two such helical HCs form the
The coiled-coil structure of the rod. The water-insoluble and salt-soluble properties of the rod region determine the salt-solubility of myosin. Roughly 500 molecules of myosin associate through their tail regions to form a dumbbell-like filament structure projecting from the head of the structure. Myosin in a thick filament associates with actin (thin filament, or F-actin) though the myosin head region. The cyclic association/dissociation of myosin and actin is determined by the ATP hydrolysis process conducted by myosin. The thin filament is a double-stranded structure of F-actin produced by polymerization of the G-actin unit.

Living animals, including fish, contain a high concentration of ATP (~ 10 mM) in muscle cells. In the relaxed state, the myosin head detaches from F-actin, thus minimizing ATP consumption and resulting in very low Mg-ATPase activity in myosin. The binding of Ca\(^{2+}\) released from the sarcoplasmic reticulum (a Ca\(^{2+}\) storage organelle) to troponin subunits (troponin-C) located on F-actin filaments triggers contraction (Ebashi et al. 1968). The binding of F-actin to myosin under these conditions significantly stimulates the Mg-ATPase activity of myosin, resulting in the production of energy sufficient for contraction. The restoration of Ca\(^{2+}\) into the sarcoplasmic reticulum relaxes the muscle.

1.3 Unstable nature of fish myosin Oxygen obtained from water by fish is transported to anaerobic muscle tissues via the blood circulation system to regenerate ATP. This oxygen supply ceases when the fish dies and consequently ATP is no longer generated in the fish muscle cells. Muscle tissues continue to consume ATP even after the death of the fish and the ATP is eventually depleted. At that time, the myosin and actin bind rigidly and irreversibly, resulting in “rigor mortis of fish”. Myosin and F-actin in dead fish muscle form a rigid complex. Fish muscle is typically processed after it is in a rigor state. Myosin in dead fish muscle is believed to be complexed with F-actin and importantly, F-actin binding to myosin results in significant stabilization. Generally, myosin in dead fish muscle remains very stable even after rigor mortis or softening by this mechanism.

It is well established that fish myosin is unstable compared with mammalian myosin. The stability of fish myosin is greatly dependent on the habitat water temperature or body temperature of the fish (Johnston et al. 1973, Hashimoto et al. 1982) (Fig. 3). This fish species-specific stability of myosin is due to an adaptation of fish to the water temperature of their environment: several amino acid residues in the myosin molecule are substituted to make the myosin suitably flexible at the water temperature preferred by that species. Muscle contraction requires suitable flexibility of the myosin structure at the fish’s habitat temperature. Accordingly, myosin from cold water fish with a low body temperature should be less rigid, while myosin from fish species living at a higher water temperature, and mammals with a high body temperature, should be much more rigid. Apparently, myosin from cold water fish is less stable than myosin from warm water fish or homiothermic animals. The unstable nature of fish myosin, especially in cold water fish species, must be overcome in utilizing fish muscle as a raw material for processed food.
2. Myofibrils: a useful material for myosin denaturation studies

2.1 Preparation of unstable fish myosin  Fish myosin is generally unstable and thus studies on its stability and suppression of its denaturation have been important research topics. The unstable nature of fish myosin prevents the application of well-established preparative methods used for myosin from mammalian tissues. For example, the purification of fish myosin using chromatographic techniques is not always successful because the myosin denatures during the chromatographic procedure, even if conducted under refrigerated conditions. Accordingly, myosin from fish species with relatively stable myosin, such as tilapia (Takashi et al. 1974), carp (Takashi et al. 1970), tuna (Murozuka and Arai 1976), and yellowtail (Kimura et al. 1977), were used in early fish myosin studies and allowed elucidation of the fundamental and structural properties of fish myosin. Preparative methods of fish myosin often include ammonium sulfate fractionation in the presence of Mg-ATP to remove actin as a precipitate using 40% saturated ammonium sulfate. Sulfate stabilizes myosin (Konno 1998) and thus myosin detached from F-actin remains stable in the presence of ammonium sulfate. However, the salt must be removed by dialysis for subsequent phases of the study. Overnight dialysis of fish myosin against an iced solution often denatures it severely. In this case, myosin must be prepared in the presence of sugar or Na-glutamate to prevent denaturation during subsequent steps (Kimura et al. 1982). Myosin and the fragment S-1 were purified from Alaska pollock muscle using sorbitol as a protectant (Konno 1992).

2.2 A myosin denaturation study performed with Mf  MF was investigated in a fish myosin denaturation study by Katoh et al. (Katoh et al. 1977). This study showed that MF is very useful and convenient to study and thus fish myosin studies based on MF are now common. The merits of using MF in fish muscle protein research are: 1) MF retains the original arrangement of myosin and actin as found in fish muscle. 2) MF can be prepared easily from a small amount of muscle tissue using simple procedures that do not require dialysis and the MF can be used the same day as its preparation. The preparative method consists of homogenizing fish muscle and repeated washing of the minced sample with a solution of neutral salts at physiological ionic strength. Homogenization destroys the muscle membranes and dissociates the muscle fibers into much finer MF fragments by predominantly tearing the long MF fibers at the Z-line, generating much shorter MF segments. The resulting MF suspension can be handled quantitatively as a solution. 3) Myosin in MF is stabilized by binding with F-actin, which decreases the possibility of myosin denaturation during subsequent preparation steps.

2.3 Non-parallel loss of myosin properties upon heating MF  Myosin exhibits ATPase activity and thus the loss of ATPase activity is a sensitive and quantitative index for accessing myosin denaturation. Myosin exhibits three types of ATPases, depending on the activating metal (Mg\textsuperscript{2+}-ATPase, Ca\textsuperscript{2+}-ATPase, and K\textsuperscript{+}-ATPase). Which is the most suitable for a myosin denaturation study? F-actin activates the Mg\textsuperscript{2+}-ATPase and suppresses the K\textsuperscript{+}-ATPase activities of myosin and thus the activities themselves do not reflect myosin denaturation. In contrast, Ca\textsuperscript{2+}-ATPase activity is not affected by the presence of F-actin and is proportional to the native myosin content of MF. Thus, Ca\textsuperscript{2+}-ATPase activity is the most commonly employed index in myosin denaturation studies (Arai et al. 1970). The habitat water temperature-dependent and species-specific thermal stabilities of fish myosin were proposed as possible indices of myosin denaturation in a study of Ca\textsuperscript{2+}-ATPase inactivation (Johnston et al. 1973, Hashimoto et al. 1982) (Fig. 3). However, since there are differences in Ca\textsuperscript{2+}-ATPase activity between fish species, Ca\textsuperscript{2+}-ATPase activity itself does not provide an index of native myosin content.

Mg\textsuperscript{2+}-ATPase activation is significantly stimulated by F-actin binding at low-ionic strength and the activation decreases with increasing ionic strength (salt concentration) due to the decreased affinity of myosin for F-actin. Moreover, Mg\textsuperscript{2+}-ATPase activation is affected by myosin filament structure: myosin in rigid filaments reacts with F-actin more efficiently than does myosin in less rigid filaments (Matsura et al. 1984, Matsuura and Arai 1986, Matsuura et al. 1988). In contrast, K\textsuperscript{+}-ATPase is inhibited by F-actin, although the inhibition mechanism is unclear. These two activities (Mg\textsuperscript{2+}-ATPase and K\textsuperscript{+}-ATPase) are used to detect structural changes in myosin filaments or F-actin denaturation in MF. For example, heating MF decreases Ca\textsuperscript{2+}-ATPase activity as the native myosin content decreases whereas the Mg\textsuperscript{2+}-ATPase activity increases (Taguchi et al. 1978, Konno and Ueda 1989) (Fig. 4). These events were explained by denaturation of the myosin tail or destruction of the myosin filament structure.

The ATPase active site locates on the myosin head region and thus Ca\textsuperscript{2+}-ATPase activity provides important information regarding denaturation of the myosin head region, but provides no information regarding denaturation of the myosin tail region. Denaturation of the myosin tail region can be detected by monitoring loss of salt-solubility because the tail region is responsible for the salt-solubility of myosin (Koseki et al. 1993). A much faster loss of salt-solubility than of Ca\textsuperscript{2+}-ATPase inactivation was observed upon heating carp MF (0.1 M KCl, pH 7.5) (Konno and Ueda 1989) (see Fig. 4), suggesting that heating MF at physiological salt concentration preferentially denatures myosin rods compared to S-1.

2.4 Chymotrypsin digestion as a useful technique to detect denaturation of the tail region of myosin  The above suggestion was confirmed by chymotrypsin digestion studies. Chymotrypsin cleaves peptide bonds at the carboxyl side of hydrophobic residues. Hydrophobic residues are usually buried inside the protein structure and thus are inaccessible to chymotrypsin when the protein is in its native conformation. Conformational change caused by heating the protein may expose susceptible residues to the enzyme, resulting in
cleavages at the newly exposed sites (Konno et al. 2000). Consequently, structural changes of proteins can be detected by their increased (or altered) digestibility, which can be analyzed easily from the SDS-PAGE (polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate) patterns of the digest. Fortunately, native myosin in its filamentous form has a single cleavage site for chymotrypsin, the S-1/rod junction (Weed and Pope 1977) (see Fig. 2). Cleavage at this site converts the myosin molecule into S-1 (head) and rod (tail) fragments. Cleavage at this site requires the prior removal of divalent metals from the Ca$^{2+}$-binding LC component of myosin that covers the cleavage site on the HC. Thus, the digestion buffer always contains EDTA to remove bound Ca$^{2+}$ from the LC. The generation of new bands in the gel electrophoresis pattern indicates additional cleavages at newly exposed sites. In other words, the decreased production of S-1 and of rods provides an index of denaturation at these two regions. Myosin contains a second chymotrypsin cleavage site within the rod region. Cleavage at this site converts myosin into heavy meromyosin (HMM) and light meromyosin (LMM), in which the S-1/rod junction is protected by adding Ca$^{2+}$ to the digestion buffer. This second cleavage site is accessible only when myosin is in the monomeric or salt-dissolved form. When dissolved myosin is digested in the presence of EDTA, it is cleaved at both sites to produce S-1, S-2, and LMM (Fig. 2). The denaturation of the S-1 and rod regions was studied using the conditions employed to cleave myosin at the S-1/rod junction. It is crucial to prevent cleavage at the second site to study rod denaturation. Conventional conditions for selective cleavage at the S-1/rod junction in mammalian myosin are 0.15 M NaCl, pH 7.5. However, these conditions are not suitable for fish myosin because fish myosin does not form rigid filaments under these conditions (Kato and Konno 1990). Thus, the NaCl concentration was reduced to 0.05 M and the pH was reduced to 7.0 to obtain selective cleavage at the second chymotrypsin cleavage site of fish Mf.

Heated Mf provided a digestion pattern different from that of native Mf due to additional cleavage at the S-2/LMM junction, suggesting that myosin filaments became less rigid upon heating Mf. Cleavage at the S-2/LMM junction resulted in a decreased amount of intact rod in the gel electrophoresis pattern. A decrease in the amount of rod produced from heated Mf correlated well with an increase in Mg$^{2+}$-ATPase activity and a decrease in salt-solubility. Chymotrypsin digestion also provides information on S-1 denaturation (Konno et al. 1990). A decrease in the S-1 HC content in the digest correlated well with Ca$^{2+}$-ATPase inactivation, which is reasonable because the ATPase active site is located in the S-1 region and inactivated S-1 would be degraded into short fragments upon digestion. A much more rapid denaturation of the rod region than of the S-1 region in Mf explains the more rapid loss of salt-solubility compared to the decrease in Ca$^{2+}$-ATPase activity.
2.5 Species-specific S-1/rod denaturation profile for fish Mf

The above conclusions regarding S-1 and rod denaturation were obtained using carp Mf. The question remains whether the observed pattern is commonly obtained with Mf from other fish species or not. It was demonstrated that the S-1/rod denaturation patterns for various fish species are different from each other. Two typical patterns are presented in Fig. 6. Heating rainbow trout Mf results in a rapid decrease in rod production whereas the amount of S-1 generated from the heated Mf remains high even after almost complete loss of rod production (Fig. 6A). This pattern is similar to that of carp Mf. On the other hand, walleye pollock Mf provided the opposite pattern: the decrease in rod content was much slower than the decrease in S-1 content. Although the data were not presented, other species gave specific intermediate patterns (Takahashi et al. 2005, Takahashi et al. 2005).

2.6 S-1/rod denaturation pattern determined from the filament structure

The S-1/rod denaturation pattern for carp Mf is altered depending in the KCl concentration and thus the myosin filament structure might affect the S-1/rod denaturation pattern (Yamamoto et al. 2002). It was reported that fish myosin tends to form less rigid filaments and their rigidity is species-specific (Matsuura and Arai 1986, Kato and Konno 1990). It is interesting to know whether the S-1/rod denaturation pattern is affected by the rigidity of the myosin filament. The rigidity of the myosin filament was altered by changing the pH of the Mf suspension from 6 to 8 (Katsura and Noda 1973, Matsuura and Arai 1986, Kato and Konno 1993). Generally, myosin forms a more rigid filament at acidic pH due to a decrease in the negative charges on myosin compared to at alkaline pH. Tilapia and catfish Mf were used in these experiments because the thermal stabilities of these two myosins are the same as that measured by Ca\(^{2+}\)-ATPase inactivation, and because these two species show different S-1/rod denaturation patterns when heated at 0.1 M NaCl, pH 7.5. Specifically, a slower denaturation of the rod region than of the S-1 region was characteristic for tilapia Mf, whereas catfish Mf showed faster denaturation of the rod region than of the S-1 region (Thavaroj et al. 2012) (Fig. 7). Changing the pH of the heating buffer altered the S-1/rod denaturation pattern for both species of fish. S-1 denaturation for both species of fish was affected similarly by changing the pH of the buffer prior to heating, and the observed S-1 denaturation was similar to the pH-dependent Ca\(^{2+}\)-ATPase inactivation profile, showing that the slowest rate of denaturation was obtained at pH 7.5 (Hashimoto and Arai 1978). However, the effect of pH on rod denaturation was different from its effect on S-1 denaturation. Rod denaturation was promoted by an increase in pH and was suppressed by a decrease in pH for both fish species. Thus, different S-1/rod denaturation patterns were obtained for different pH values of the buffer used to prepare the samples prior to heating. When heated at an acidic pH such as pH 6, S-1 denaturation was accelerated and rod denaturation was suppressed and the gel electrophoresis pattern was consistent with much faster denaturation of S-1 than of rod. In contrast, heating at an alkaline pH such as pH 8 promoted rod denaturation but had less of an effect on S-1 denaturation, and the gel electrophoresis pattern was
consistent with much faster denaturation of rod than of S-1. In other words, the S-1/rod denaturation pattern is not fixed for a given fish species and is affected significantly by the pH of the environment or by the filament structure. Apparently, the S-1/rod denaturation profile for tilapia Mf at pH 7.0 is similar to that of catfish Mf at pH 6.0. Similarly, the profile for catfish Mf at pH 7.5 resembled the profile at pH 8.5 for tilapia Mf. It is well known that the filament-forming ability of fish myosin is species-specific (Matsuura et al. 1988). The S-1 and rod denaturation profiles obtained when Mf in 0.1 M NaCl, pH 7.5, is heated provides information on the rigidity of myosin filament: faster rod denaturation is an index of a less rigid filament structure. Heating Mf at pH 7.5 is a suitable pH condition for distinguishing the myosin S-1/rod denaturation profiles of various fish species (Fig. 6).

3. F-actin denaturation in Mf upon exposure to high concentrations of neutral salt.

It is generally believed that F-actin is much more thermostable than myosin in Mf. Heating Mf in a manner to completely denature myosin usually does not denature the F-actin component. However, the unstable nature of F-actin in Mf was discovered by Wakameda et al. (Wakameda and Arai 1984) who demonstrated that the exposure of carp Mf to a high concentration of neutral salt, such as 2 M NaCl or KCl, denatures F-actin preferentially. This high concentration of salt does not denature myosin itself, and thus the myosin in salt-treated Mf loses the protection of F-actin, making the myosin very unstable. Consequently, the stability of myosin in Mf in the presence of 1.5 – 2 M NaCl or KCl mirrors the stability of isolated myosin. The rate of thermal inactivation of Mf ATPase in the presence of 0.1 M and 2 M KCl provides information on how strongly F-actin stabilizes myosin in Mf. The extent of stabilization is different between fish species (Wakameda et al. 1987).

F-actin stabilizes myosin in the Mf of some fish species (e.g., white croaker) by as much as 200 times, while for other species (e.g., valleyle pollock) myosin is stabilized by F-actin only 10 – 20 times. F-actin denaturation can be observed even if the sample is kept on ice and native myosin is liberated from the denatured actin (Wakameda and Arai 1986). The denatured actin produced remains water-soluble and thus first the F-actin is disassembled into denatured G-actin (Wakameda and Arai 1985). It is reported that myosin binding protects actin from salt-induced denaturation in Mf. Actomyosin or salt-dissolved Mf is usually stored in a medium containing 0.5 M NaCl or KCl in which practically no actin denaturation is detected. However, the addition of Mg-ATP (or Mg-pyrophosphate) to the actomyosin solution caused actin denaturation (Yagi and Arai 1986, Matsukawa et al. 1990). Ammonium sulfate fractionation in the presence of Mg-ATP is used to remove actin from myosin during myosin preparation. F-actin precipitated using 40% saturated ammonium sulfate is denatured, probably due to exposure to this high concentration of ammonium sulfate. However, the same concentration of ammonium sulfate added to Mf dissolved in 0.5 M KCl in the absence of Mg-ATP did not denature F-actin (Torigai and Konno 1997, Torigai and Konno 1997). These findings indicate that myosin binding protects F-actin from denaturation by salt.

4. Myosin and actin denaturation by freezing Mf and fish muscle

4.1 Actin denaturation detected in frozen stored fish Mf

Freezing is used to minimize the loss of quality of fish muscle for extended periods of time. The extent of myosin denaturation during the storage of frozen fish is often estimated using Mf as a model. Many early studies were conducted using salt-dissolved actomyosin. The index commonly employed to detect myosin denaturation was the loss of ATPase activity (Arai et al. 1970, Noguchi and Matsumoto 1970, Noguchi and Matsumoto 1971, Inoue et al. 1992). However, no information is available on rod denaturation during the frozen storage of Mf and thus experiments using chymotrypsin digestion were conducted. The denaturation of frozen myosin was characterized by heating myosin and comparing it to flounder Mf (0.1 M NaCl, pH 7.5). Parallel denaturation of the S-1 and rod regions of myosin was characteristic for flounder Mf upon heating and this profile was similar to that of yellowtail Mf (Takahashi et al. 2005). The storage of frozen Mf (0.1 M NaCl, pH 7.5) at -20°C resulted in a gradual decrease in myosin Ca\(^{2+}\)-ATPase activity over one week and the decrease was correlated with S-1 denaturation, as revealed by chymotrypsin digestion experiments. However, the amount of rod produced in frozen stored Mf in which the ATPase had been inactivated was practically the same as the amount of rod produced in unfrozen Mf, demonstrating that frozen storage of Mf did not damage the tail portion of myosin.

Chymotrypsin digestion unexpectedly revealed that the intensity of the actin band observed upon gel electrophoresis of the digest decreased drastically upon frozen storage of Mf, demonstrating actin denaturation upon frozen storage of Mf at 0.1 M NaCl (Fig. 8). Actin denaturation was not observed upon heating Mf, thus actin denaturation is characteristic of frozen Mf. Moreover, actin denaturation proceeded at a rate similar to that of Ca\(^{2+}\)-ATPase inactivation and the decrease in S-1 content. A possible mechanism explaining these observations is that myosin is denatured as a result of loss of protection by actin, or that actin denaturation determines myosin denaturation in Mf. The mechanism was investigated further using tilapia Mf because tilapia myosin is much more stable than flounder myosin. Frozen storage of tilapia Mf (0.1 M NaCl, pH7.5) resulting in the denaturation of actin, similar to that in flounder Mf, whereas S-1 denaturation and Ca\(^{2+}\)-ATPase inactivation in frozen tilapia was much slower than actin denaturation, indicating that tilapia myosin dissociated from denatured actin slowly. Actin denaturation in tilapia Mf was also detected when stored at -40°C. There are reports of actin denaturation upon frozen storage of carp...
actomyosin, in which actomyosin in 0.6 M KCl was stored at 
and this was explained by salt-induced actin denaturation due to 
the condensation of KCl upon freezing the solution at −10°C. 
Similar actin denaturation was observed even in 0.1 M NaCl and 
−20°C. What is the mechanism underlying actin denaturation at 
these conditions? Is salt condensation involved in the mechanism? 
To answer these questions, Mf was suspended in solutions 
containing different NaCl concentrations and then stored frozen. 
When stored in 10 – 20 mM NaCl, actin denaturation was no longer 
observed, demonstrating that the NaCl concentration is a crucial 
parameter in actin denaturation in frozen Mf. Indeed, severe actin 
denaturation was observed at 0.1 M NaCl and the maximum effect 
was obtained at around 0.2 M NaCl. It was concluded that salt 
condensation is the mechanism by which actin is denatured in 
frozen Mf.

4.2 Comparison of actin and myosin denaturation between Mf 
and fish muscle upon frozen storage

Given the above actin denaturation mechanism, the question could be raised whether such 
actin denaturation is detectable when fish muscle is stored frozen 
because fish muscle cells contain salt concentrations similar to 
those used in the above experiments. Myosin and actin denaturation 
in frozen-stored fish muscle was analyzed by converting the muscle 
into muscle homogenate. Frozen storage of flounder homogenate 
at −20°C decreased the Ca^{2+}-ATPase activity very slowly, with 
70% of the activity remaining after 60 days (Fig. 9) whereas only 
70% of the activity of Mf remained after 2 – 3 days. Moreover, 
chymotrypsin digestion revealed no actin denaturation in the frozen 
stored homogenate. Consequently, myosin in frozen fish remained 
stable by forming a strong complex with native actin. The 
difference in actin denaturation between Mf and fish muscle would 
be due to a process by which the salt solute freezes. Assuming the 
Mf suspension in 0.1 M NaCl contained 2 mg/mL of protein, the 
process of freezing the water in the solution would expel a large 
amount of salt together with the protein. The resulting high NaCl 
concentration would be sufficient to denature actin in the 0.1 M 
solution. When fish muscle is frozen, water in the muscle cells 
freezes and NaCl condensation is apparently insufficient to cause 
actin denaturation. Similar actin denaturation was reported with 
kuruma shrimp (Jantakoson et al. 2013), suggesting that actin 
denaturation upon freezing is a commonly observed event when 
Mf is frozen.

The factors that determine myosin denaturation upon the 
frozen of fish muscle are the freezing temperature, the freezing 
rate, and the storage temperature. Fukuda et al. carefully designed 
an experimental plan to elucidate the roles of these factors in 
myosin denaturation using Chum mackerel mince. Ca^{2+}-ATPase 
activity provided the myosin denaturation index (Fukuda et al. 
1984) (Fig. 10). The conclusion from these experiments was that 
the extent of myosin denaturation is mainly determined by the 
storage temperature rather than the freezing temperature or freezing 
rate. Although there are many reports on the denaturation of 
myosin in fish muscle during freezing, few of the experiments 
described in these reports were designed more carefully than those 
by Fukuda et al.

The rate of denaturation obtained by heating Mf was practically 
the same as that obtained by heating fish muscle and is due to actin 
not being denatured in heated Mf. Fortunately, Mf is appropriate 
for studying the thermal denaturation of myosin in fish.

5. Quality evaluation of frozen surimi, an intermediate 
material for thermal gel production.

5.1 Suppression of myosin denaturation in frozen surimi by the 
addition of sugar

Frozen surimi was developed in the 1960s in
Hokkaido using walleye pollock. The product is a successful example of a value-added product manufactured from less valuable resources because the fish used is mainly captured for its roe and not for consumption of the fish itself. The processing of frozen surimi involves washing minced walleye pollock with chilled water, dewatering the washed mince, the addition of sugars or sugar alcohols as cryoprotectants to the mince, and finally freezing and storage (Arai et al. 1970). Sugars and/or sugar alcohols are essential for preventing myosin denaturation during the frozen storage of surimi (Arai et al. 1970, Noguchi and Matsumoto 1970, Noguchi and Matsumoto 1971, Matsumoto and Arai 1985, Matsumoto and Arai 1986). Unstable walleye pollock myosin in frozen surimi was kept in its native state in the presence of sugar for an extended period of time (at least one year). Once the principles underlying the production of frozen surimi were established, Japanese fishery companies sent their factory ships to walleye pollock fishing grounds to produce much higher quality surimi on-ship by using much fresher fish.

The quality of frozen surimi has been evaluated by its gel-forming ability because surimi is an intermediate material for thermal gel production. It is well recognized that myosin in fish muscle or in frozen surimi is responsible for the gel formation in fish muscle or in frozen surimi, and that denatured myosin does not produce an elastic gel. Therefore, surimi produced on factory ships from very fresh fish is generally graded to be of high quality whereas surimi produced from less fresh fish on land is referred as low quality surimi.

5.2 Total ATPase activity as a quality index of frozen surimi

The first attempt to evaluate the quality of surimi using a biochemical method was by Kawashima et al. in 1973 (Kawashima et al. 1973, Kawashima et al. 1973). Their idea was that the quality of surimi can be expressed by its native myosin content. They used Ca$^{2+}$-ATPase activity as the index of native myosin content. Their original method involved extracting actomyosin from surimi using 0.6 M KCl, then measuring the Ca$^{2+}$-ATPase activity and total protein content in the extracted actomyosin. They obtained the value of the index (total ATPase activity) by multiplying the Ca$^{2+}$-ATPase activity by the total protein content of the surimi. Using this index, they studied the protective effect of cryoprotectants on myosin in surimi. Katoh et al. simplified the method by using Mf instead of actomyosin, and this simplified method does not require extraction or dialysis (Katoh et al. 1977). A correlation between the total ATPase activity and gel strength for various kinds of surimi samples was proposed and these two parameters gave a positive correlation. Using the same idea of total ATPase activity, we simplified the measurement into two steps: sample preparation from surimi, and Ca$^{2+}$-ATPase activity measurement. Surimi homogenate was prepared by homogenizing surimi in a fixed volume of 0.1 M NaCl, pH 7.5, and then Ca$^{2+}$-ATPase activity was assayed and the protein content was measured. Water soluble components had been removed from the sample during the leaching step in surimi production and thus repeated washing procedures to prepare Mf were not required. Sugars and sugar alcohols added to the surimi did not affect the measurements. Ca$^{2+}$-ATPase activity was measured using a pH stat apparatus rather than the conventional colorimetric measurement of liberated inorganic phosphate (Pi). The principle of the pH stat method is that a drop in pH caused by an increase in H$^+$ concentration resulting from Pi released due to ATP hydrolysis can be detected using a pH meter, and the drop can be compensated for by adding an alkaline solution. Consequently, the amount of NaOH solution required to neutralize the H$^+$ parallels ATP hydrolysis.

5.3 Quality evaluation of various grades of surimi by measuring total ATPase activity

Frozen surimi is graded by the producers by considering the material used in its production, especially the number of days since the fish were caught and the processing method. Traditionally, surimi produced on-ship is graded higher than land processed surimi due to the freshness of the fish. The total ATPase activities for commercially distributed frozen surimi produced in Alaska and in Hokkaido were analyzed using the pH stat method. The surimi produced in Hokkaido was land-processed 2nd grade, while the surimi produced in Alaska was graded differently. The SA grade is believed to be the highest quality, followed by the FA and KA grades. The results indicated that land processed 2nd grade surimi tended to show low ATPase activity while the surimi produced in Alaska (SA, FA, and KA) tended to show high activity. This commercial grading reflected well the native myosin content measured as total ATPase activity. The protein content of surimi did not reflect the quality of the surimi, thus clearly demonstrating that the native myosin content accessed by ATPase activity is a good index of the quality of surimi (Koseki et al. 2005, Koseki et al. 2005) (Fig. 11).


6.1 Role of temperature and pH on myosin denaturation in
tuna muscle  The previously described method for evaluating the quality of frozen surimi was successfully applied to evaluate the quality of Bluefin tuna. Tuna is usually auctioned as whole fish without knowing the quality of the muscle. Substandard tuna muscle is termed “YAKE-muscle (YAKE-NIKU)” and is often sold at fish markets. The term “YAKE-NIKU” is due to the cooked-like appearance of the tuna and is characterized by its faded whitish color and low elasticity, low water retention, and its bitter or sour taste. Such tuna is not suitable for consumption as sashimi. “YAKE-NIKU” has a low pH due to the accumulation of lactic acid produced by the anaerobic metabolism of glycogen and the high body temperature of the tuna caused by vigorous movement during the catch (Konagaya et al. 1969, Konagaya 1977, Konagaya and Konagaya 1979). Tuna may retain a high temperature for a longer time than other fish during the cooling process because its large size prevents rapid cooling.

6.2 Role of heating in YAKE-muscle  Normal Bluefin tuna sold in markets has a low pH of around 5.4 – 5.7. This decreased pH alone does not cause YAKE; as explained above, high temperature of the tuna muscle is also involved. Myosin denaturation was not detected in normal tuna muscle showing a low pH as judged by Ca\textsuperscript{2+}-ATPase activity, salt-solubility of the myosin, and S-1/rod production, suggesting that such tuna was cooled properly. To understand the role of exposure to high temperature in the generation of YAKE muscle, normal tuna exhibiting a low pH was heated at 30°C, a temperature that tuna might be exposed to when captured. Upon heating for 40 – 60 min, the color of the tuna sample changed from brilliant red to faded brown, similar to the color generally reported for YAKE-NIKU tuna in fish markets. Severe myosin denaturation was also observed in the heated tuna sample. Thus, a combination of high body temperature (e.g., 30°C) and decreased pH (e.g., 5.5) cause YAKE in caught Bluefin tuna.

Myosin denaturation was analyzed using naturally occurred YAKE-NIKU Bluefin tuna. The YAKE characteristic of an abnormal color was detected easily with the naked eye. This abnormal color was restricted to the deepest portion of the fish flesh, near the spine. The outer part of the flesh looked normal. Myosin in the YAKE region had already been denatured while no myosin denaturation was found in the outer part of the same tuna (Konno and Konno 2014).

6.3 Simulation proposes the importance of the initial cooling step in preventing myosin denaturation in tuna  The above observations indicated that suppressing a rise in body temperature, or quick cooling of fish, are key techniques for preventing YAKE. Knowledge of myosin denaturation rates under various conditions is required to predict the occurrence of YAKE. First, myosin denaturation rates at fixed temperature but various pH values were measured and the measurements were also conducted while changing the temperature; in each case, the concentration of Mf from Bluefin tuna was determined. The pH-dependent inactivation rates at 1°C intervals were measured, allowing calculation of the myosin denaturation rates for any combination of temperature and pH (Fig. 12). Given the cooling process used for caught tuna, several factors that determine myosin denaturation must be considered: the initial body temperature of the tuna, the cooling rate, and the profile of the pH drop. An example of a simulated myosin denaturation profile during the cooling process is shown in Fig. 13. Myosin denatures significantly when the initial body temperature is high, such as 30 or 33°C, while very little myosin denaturation occurs when the body temperature is 23°C. The obvious conclusion is to suppress the increase in body temperature of the tuna during the catch if possible. It is also clear that myosin denaturation is significant during the early phase but very little myosin denaturation occurs in the latter phase of the simulation. Essentially no myosin denaturation was observed when the temperature was below 20°C under any condition. In other words, to prevent YAKE in tuna, the most important point is to cool the fish below 20°C as quickly as possible.

7. Characteristic denaturation profile of myosin in squid mantle muscle

7.1 Suppressive effect of Ca\textsuperscript{2+} on squid myosin  Squid, shellfish, shrimp, and crab are important marine resources. Squid mantle muscle is an obliquely striated structure and is different from the striated structure of fish dorsal muscle. Squid muscle contains a different muscle contraction regulation system, the so-called myosin-linked regulation commonly observed in mollusk muscle such as scallop adductor muscle (Kendrick-Jones et al. 1970). In this system, muscle contraction is triggered by direct binding of Ca\textsuperscript{2+} to a myosin light chain component (LC) termed the regulatory light chain (RLC) (Konno 1978). The receptor for Ca\textsuperscript{2+} in fish or mammalian muscle is troponin located on F-actin.

![Fig. 11. Total Ca\textsuperscript{2+}-ATPase activity for commercial frozen surimi of walleye pollock with various grades. Total ATPase activity was calculated by measuring Ca\textsuperscript{2+}-ATPase and protein content for surimi. Alphabets and 2 are commercial grades given by the producers of the surimi samples.](image-url)
filaments and is responsible for actin-linked regulation (Ebashi et al. 1968).

Myosin denaturation in squid Mf is unique. For example, squid myosin in Mf, namely myosin bound with F-actin, is significantly stabilized in the presence of divalent metal such as Ca\(^{2+}\). The stabilizing effect of Ca\(^{2+}\) was also detected with squid myosin but the magnitude of the effect was much smaller (approximately 3 times) (Ohno et al. 2011). F-actin binding to squid myosin increased the stabilizing effect of Ca\(^{2+}\). The stabilizing effect of Ca\(^{2+}\) is also observed with squid myosin S-1 lacking the Ca-binding LC, indicating that the mechanism does not involve Ca\(^{2+}\) binding to the RLC. Moreover, the Ca\(^{2+}\) concentration required for the stabilization is much lower (10 nM) than that required for regulating muscle contraction mediated through Ca\(^{2+}\) binding to the RLC. The Ca\(^{2+}\) concentration in the relaxed state of muscle is sufficiently high for stabilization. Accordingly, myosin in squid muscle is always stable irrespective of the relaxation and contraction of the muscle. The stabilizing effect of Ca\(^{2+}\) on squid myosin denaturation was observed for all squid species examined (Konno 1991) whereas the stabilizing effect by Ca\(^{2+}\) is not always observed with other mollusk Mfs. For example, the stability of myosin in scallop adductor muscle Mf was not affected by Ca\(^{2+}\) or EDTA addition. Other mollusk Mfs, such as surf clam foot muscle and abalone foot muscle Mf, were stabilized by the presence of Ca\(^{2+}\) but the magnitude of the stabilization was smaller than that found with squid Mf.

7.2 Response of squid myosin to high concentrations of salt
The second factor that affects the stability of myosin in squid Mf is salt concentration (Fig. 15). As mentioned above for fish Mf, myosin in Mf is stabilized upon binding with F-actin under physiological ionic strength conditions and this stabilization is lost at high concentrations of salt. Like fish Mf, the Ca\(^{2+}\)-ATPase inactivation rate for squid Mf increased with increasing KCl concentration. However, the maximum rate was obtained at approximately 0.75 M KCl, which is roughly half the concentration required to inactivate fish Ca\(^{2+}\)-ATPase. This result indicated that the stabilizing effect of F-actin was eliminated at a lower
concentration of salt for squid Mf (Wakameda and Arai 1984). Usually, 0.5 – 0.6 M KCl (or NaCl) is used to dissolve fish and squid Mf. Squid myosin in Mf dissolved by the addition of 0.5 M NaCl is unstable due to loss of protection by F-actin. An increase in KCl (NaCl) concentration from 0.1 to 0.5 M does not accelerate myosin denaturation using fish Mf (Fig. 15B), so significant destabilization upon dissolving squid Mf in 0.5 M salt was not expected. The denaturation rates of squid Mf at 0.1 M and 0.75 M KCl provided the same degree of stabilization as that provided by F-actin. (roughly 500 times) (Fig. 15A), which is the highest stabilization observed for any Mf studied, including fish Mf. The extent of stabilization for fish Mf, calculated from the rates at 0.1 M and 1.5 M KCl, was less than that for squid Mf (Fig. 15B).

The muscle portion of a marine resource is the most important food component. Myosin is the major protein in muscle and thus the idea that the quality of the muscle is determined by the quality of the myosin seems scientifically reasonable. Thus, the denaturation of myosin in different marine resources caused by different factors has been studied. The term “myosin denaturation” is easy to use but difficult to define because myosin is multifunctional. The author has extensively studied myosin denaturation and has proposed new ideas and useful techniques to better understand myosin denaturation. Fundamental to this is how to detect changes in the biochemical properties of myosin upon various treatments. We utilize various marine resources as food, and a principle developed using one species cannot be applied to other species directly. For example, the information accumulated regarding the properties of fish myosin cannot be applied to squid myosin. Therefore, it is important to understand the differences and similarities of muscle proteins between species. The author hopes that this review is helpful for readers involved in the utilization of marine muscle proteins as food.

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