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Preceded actin denaturation that accelerates myosin denaturation in frozen stored tilapia myofibrils

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Abstract Myosin and actin denaturation in tilapia myofibrils (Mf) (0.1 M NaCl, 20 mM Tris-HCl, pH 7.5)) during its frozen storage at -10, -20, and -40 °C was studied. Ca^{2+}-ATPase inactivation at -10 °C was fast, while one at -20, and -40 °C was very slow. Myosin kept its solubility into 0.5 M NaCl even after the inactivation. Amount of subfragment-1 (S-1) generated from Mf by chymotryptic digestion decreased similarly to Ca^{2+}-ATPase inactivation. Amount of rod produced from the frozen stored Mf was kept high, which explained high salt-solubility of myosin. Actin denaturation in frozen stored Mf was revealed by chymotrypic digestion showing a decreased remaining actin content in the digest. Actin denaturation was much faster than Ca^{2+}-ATPase inactivation. When tilapia meat was frozen stored, Ca^{2+}-ATPase inactivation was very slow and the difference in the rates for these at -10 °C was about 9 times slower. Moreover, practically no actin denaturation was found in the frozen stored meat. It was concluded that native actin in frozen meat protects myosin from denaturation during the storage but such protection by actin was no longer expected for Mf due to quick actin denaturation. Thus the Ca^{2+}-ATPase inactivation rate obtained with Mf represented that of myosin itself with no protection by actin. Thus the rate obtained with Mf should not be applied to one in frozen meat. Mf suspended in 0.1 M NaCl is not a suitable model material to obtain myosin denaturation rate in frozen fish meat.

Keywords tilapia, myofibrils, actin denaturation, myosin, frozen storage

Introduction

In general, myosin denaturation affects the quality of fish meat and meat-based product such as frozen Surimi. Unstable nature of walleye Pollock myosin is overcome by adding sugar or sugar alcohol as chryoprotectant [1]. Consequently, frozen surimi keeps its gel forming ability at least for one year when stored at about -20°C. Frozen fish fillet is also world-widely distributed. Many papers have been published to explore freeze denaturation of myosin by using various kinds of material such as salt-dissolved actomyosin, myofibrils (Mf), and fish fillet [2-9]. Among the materials, Mf is the most popularly used one because it keeps intact arrangement of myosin and actin filaments and can be handled very quantitatively like solution. Suppressive effect of various types of organic compopunds including sugars and sugar alcohols on myosin denaturation was quantitatively studied by using Mf suspended in 0.1 M KCl [4]. Myosin denaturation by monitoring various properties of myosin during frozen storage of carp Mf was studied [9] and myosin subfragment-1 of carp as a model material [10]. In the former paper, myosin denaturation caused by frozen storage was different from one by heating [9]. In the latter paper, less severe damages on S-1 molecule was reported.

Importance of frozen storage temperature rather than freezing temperature in myosin denaturation was carefully studied by Fukuda et al. [8]. They stored fish fillet by changing freezing and storage temperatures and myosin denaturation under these conditions was studied by measuring Ca^{2+}-ATPase activity.

In fish muscle after rigor mortis, myosin forms very strong complex with actin that leads stabilization of myosin. Myosin in Mf is also in stable state as a consequence of complex formation with actin in the absence of Mg-ATP. Regarding to stability of actin in Mf, it is believed that actin is very stable and its binding to myosin stabilizes myosin [11]. Moreover, it is reported that F-actin from various species of fish and mollusk similarly stabilizes myosin of various species [12]. Thermostable actin is not resistant to high concentration of neutral salt such as 1.5 M KCl or NaCl [13]. Selective actin denaturation by treatment of Mf with high concentration of neutral salt at low temperature releases myosin from denatured actin in Mf [14].

F-actin denaturation during frozen storage of carp actomyosin dissolved in 0.5 M KCl was reported [7]. As the event was the most significant at -10 $^{\circ}$C, the actin denaturation was explained by condensation of KCl in unfrozen medium at the temperature. Since then, Mf at 0.1 M NaCl (or KCl) and storage temperature of -20 $^{\circ}$C are usually used for studying myosin denaturation during frozen storage, where no salt condensation is expected. Recently, we found that actin in kuruma prawn Mf denatured very quickly upon its frozen storage [15]. A similar actin denaturation was also detected with frozen stored flounder Mf [16]. In these papers, chymotryptic digestion was the technique to detect actin denaturation in Mf. In both cases, Ca^{2+}-ATPase inactivation and actin denaturation proceeded at the same rate.

In the present study, we confirmed actin denaturation in frozen stored tilapia Mf and its denaturation rate was compared with that of myosin to answer the question whether actin denaturation determines myosin denaturation. As tilapia myosin is one of the most stable

fish myosin so far [17], myosin denaturation after the actin denaturation was expected if actin denaturation is quick. To understand the stability of myosin in frozen stored meat itself, actin and myosin denaturation in frozen stored tilapia meat was also studied.

Materials and methods

Mf preparation

Tilapia *Oreochromis niloticus* with a length of roughly 10 cm cultured in Hakodate campus, Hokkaido University was used. Mf was prepared from its dorsal muscle as reported elsewhere [18] by washing the homogenized muscle with 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5) repeatedly.

Frozen storage of Mf and meat block

Mf put in test tubes suspended in the above medium was stored in freezers at -10, -20, and -40 °C. The Mf concentration used was commonly 2.5 mg/ml. Fish dorsal muscle block (about 5 g) was wrapped with plastic film and stored in the same freezers at various temperatures. Frozen stored meat was quantitatively converted into muscle homogenate [19]. Chopped meat (1 g) was taken in 50 ml disposable plastic centrifuge tube and suspended in 40 ml of 0.05 M NaCl, 20 mM Tris-maleate (pH 7.0). The mixture was centrifuged at 3,000 xg for 5 min to collect washed mince. The residue obtained was

homogenized on Polytron PT 3100 (Kinematica AG, Littau Switzerland) for 30 sec 4 times at the speed of 15,000 rpm in 20 ml of the above solution. To the above muscle homogenate, 20 ml of the same solution was added and finally filtered through a layer of coarse plastic mesh (1 mm width) to remove connective tissue. The homogenate was handled quantitatively like Mf.

Indices to detect myosin and actin denaturation in Mf and in meat

Myosin denaturation in Mf and in muscle homogenate was studied by monitoring the following changes; Ca^{2+}-ATPase activity, salt soluble myosin content in the presence of 1 mM Mg-ATP, monomeric myosin content as measured by ammonium sulfate fractionation in the presence of 1 mM Mg-ATP, and the amount of S-1 and rod produced by chymotryptic digestion as previously reported [11, 20, 21]. Actin denaturation was accessed by the decreased density of actin band in the chymotryptic digests. Chymotryptic digestion was performed for 60 min at 20°C in a digestion medium of 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) and 1 mM EDTA using 1/400 (w/w) of chymotrypsin over Mf or homogenate proteins. SDS-PAGE (Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate) was conducted as reported [22].

Results

Myosin denaturation in frozen stored tilapia Mf

Ca^{2+}-ATP inactivation has been used as a sensitive index to monitor myosin denaturation [1]. Changes in Ca^{2+}-ATPase activity during the frozen storage of tilapia Mf at -10, -20, and -40°C were followed (Fig. 1). Activity decrease was very slow when stored at -20 and at -40°C and the samples stored for 16 days still showed the remaining activity of 93 and 96%, respectively. Ca^{2+}-ATPase inactivation at -10°C was obvious and the activity dropped to 61% in 16 days. It was confirmed that lowering frozen storage temperature reduces myosin denaturation rate. The Ca^{2+}-ATPase inactivation found with tilapia Mf at -20°C was much slower than that with flounder Mf [16] and kuruma prawn Mf [15].

Myosin denaturation in frozen stored Mf was further studied by monitoring changes in several properties. As the progress of Ca^{2+}-ATPase inactivation at -20 and -40°C was very slow, myosin denaturation in the samples stored at -10°C was studied. The properties monitored are salt-solubility measured in the presence of Mg-ATP, namely solubility of myosin in detached form from F-actin and monomeric myosin content recovered in the supernatant at 40% saturated ammonium sulfate in the presence of Mg-ATP (Fig. 2a). Ca^{2+}-ATPase inactivation was also shown in the same figure. Salt-solubility and monomeric myosin content decreased more slowly than ATPase inactivation. The results indicated that inactivated myosin in Mf still retained its salt solubility and was not in aggregated form.

As the salt-solubility of myosin and Ca^{2+}-ATPase inactivation occurred differently upon frozen storage of Mf, we further studied myosin denaturation at subfragment-1 (S-1), the region for ATPase activity and rod, the regions for salt solubility of myosin molecule by using chymotryptic digestion technique (Fig. 3) [11]. The conditions for digestion are

144 favorable to cleave myosin into S-1 and rod. Control Mf before frozen storage showed a
145 selective cleavage into S-1 and rod as expected (Fig. 3a, 0 min). The digestion patterns for
146 the frozen stored Mf at -10°C showed a decreased production of S-1 (Fig. 3a). Amount of
147 S-1 produced decreased gradually with the storage period, while the amount of rod
148 produced was kept high throughout the storage period. It was concluded that frozen storage
149 of Mf damaged S-1 portion but rod portion was kept native irrespective of S-1 denaturation.
150 Although the data were not presented, a majority of rod produced as well as S-1 was
151 recovered in the supernatant at 40% saturated ammonium sulfate suggesting that myosin
152 tail portion was kept in monomeric form during the storage. The amount of S-1 and rod
153 produced were measured and shown in Fig. 2b. Decreases in the amount of S-1 and rod
154 production upon frozen storage were similar to ATPase activity decrease and salt-solubility,
155 respectively. It was demonstrated that S-1 denaturation determined remaining ATPase
156 activity and that of rod denaturation determined salt solubility of myosin. Thus, slow rod
157 denaturation was the reason for high salt solubility of myosin even after Ca^{2+}-ATPase
158 inactivation.
159
160 Actin denaturation in frozen stored tilapia Mf
161
162 The chymotryptic digestion of frozen stored Mf also provided the information on actin
163 denaturation (Fig. 3a). Actin in Mf before frozen storage was resistant to the chymotryptic
164 digestion and actin content in the digest was similar to one in intact Mf. However, actin
165 content in the chymotryptic digest decreased significantly when frozen stored Mf was used

166 indicating that a majority of actin molecule in Mf was degraded into much shorter
167 fragments by the digestion (Fig. 3a). Frozen storage of Mf only for 2 days reduced
168 remaining actin content in the digest significantly. Indeed amount of S-1 decreased
169 gradually upon frozen storage of Mf, the decrease of actin content was much faster than it.
170 Thus, preceded actin denaturation in frozen stored Mf was demonstrated. Remaining actin
171 content in the digest was measured and compared with S-1 content (Fig. 2b). Storage for 2
172 days decreased actin content to 25%, while amount of S-1 produced was as high as 92%.
173 As such quick actin denaturation in Mf was found at -10°C, effect of storage temperature
174 on it was further studied by lowering temperature to -20 and -40°C. A clear actin
175 denaturation as studied by chymotryptic digestion was shown in Figs. 3b and 3c. It was
176 clear that lowering storage temperature practically did not prevent actin denaturation. Actin
177 denaturation at -40°C seemed a little slower than that at -10°C (Figs. 3a, c). In this
178 experiment, Mf suspended in 0.1 M NaCl not 0.6 M was used and storage temperature was
179 set to -20 and 40°C. Nevertheless actin denaturation was observed there. Thus, salt
180 condensation was not the reason for actin denaturation in frozen stored Mf. We have
181 already reported actin denaturation found with kuruma prawn [15] and flounder Mf [16] at
182 -20°C. Actin denaturation observed with tilapia Mf was clearly different from one with two
183 Mf samples, i.e., actin denaturation found with tilapia Mf was much faster than myosin
184 denaturation. This was a contrast to the cases of actin denaturation in prawn and flounder
185 Mf, in which myosin denaturation as studied by Ca^{2+}-ATPase inactivation and actin
186 denaturation proceeded at the same rate.
187

188 Myosin and actin denaturation in frozen stored tilapia muscle

189

190 Preceded actin denaturation to myosin denaturation was proved when Mf at 0.1 M NaCl
191 was frozen stored. We studied whether actin denatures when tilapia meat was frozen stored.
192 Small pieces of tilapia meat (~5 g) wrapped with plastic film were stored in the same
193 freezer at -10, -20, and -40 °C up to 170 days. Myosin and actin denaturation in the frozen
194 meat was studied by using muscle homogenate prepared from the frozen stored meat.
195 Ca^{2+}-ATPase inactivation at these temperatures was followed and the profiles were shown
196 in Fig. 4. In the same figure, Ca^{2+}-ATPase inactivation for Mf at -10 °C was also showed
197 for comparison. Ca^{2+}-ATPase inactivation progressed very slowly during the frozen storage
198 and the inactivation was temperature dependent. The inactivation at -10 °C was the fastest
199 among the temperatures (Fig. 4). It was clear that inactivation for myosin in meat was much
200 slower than that in Mf. For example, frozen storage of Mf at -10 °C for 16 days decreased
201 the activity to 60%. The same decreased was found in 110 days for frozen stored meat. The
202 inactivation rate for Mf was calculated to be 2.4×10^{-2} day^{-1} and one for meat was $4.2 \times$
203 10^{-3} day^{-1}. Myosin in meat was proved to be 9 times stable that that in Mf. In other words,
204 myosin denaturation study by using Mf cannot be applied directly.
205 Myosin denaturation in frozen stored meat was also studied by measuring salt-solubility
206 and monomeric myosin content (Fig. 5a). Salt-solubility and monomeric myosin content
207 decreased more slowly than Ca^{2+}-ATPase inactivation. Relative changes of these three were
208 generally the same for frozen stored Mf (see Fig. 2a).
209 Myosin denaturation at S-1 and rod regions as well as actin denaturation in frozen meat was

studied by using chymotryptic digestion. The digestion pattern for the meat before storage and ones for meat stored for 170 days at three temperatures were compared in Fig. 6. Meat before frozen storage showed two S-1 and rod bands in the chymotryptic digest pattern. Actin in the sample was resistant to the digestion (Fig. 6a). Relative density of S-1 to rod was also the same as for Mf. Frozen stored meat at -10°C for 160 days gave a decreased amount of S-1 to that of rod (Fig. 6b). Actin in the sample was similarly resistant to chymotryptic digestion. Frozen storage at all temperatures for 170 days did not change actin content in the digest, while S-1 content decrease was observed with the sample stored at -10°C (Fig. 6b). Amounts of S-1, rod, and remaining actin content in the digests were estimated and presented in Fig. 5b. As proved with frozen stored Mf, amount of rod content decreased slowly than S-1. Slow loss of salt-solubility was explained by slow rod denaturation. S-1 denaturation explained Ca^{2+}-ATPase inactivation. These conclusions were the same as led with frozen stored Mf. There was practically no decrease of remaining actin content during the storage indicating that actin denaturation in frozen stored meat was negligible. It was concluded that actin in meat is very stable and consequently myosin in meat is well stabilized by actin. It was also concluded that Mf is not proper material to study myosin denaturation in frozen meat. Myosin rod region was proved to be resistant to freezing and its native structure was kept for a long period, which keeps high salt-solubility of myosin.

By measuring several changes of myosin properties, it was concluded that Ca^{2+}-ATPase inactivation was the most sensitive index to monitor myosin denaturation in frozen stored meat.

Discussion

Mf has been used as a useful material for studying myosin denaturation in fish meat because Mf keeps intact arrangement of myosin and actin filaments as in muscle and the Mf suspension can be handled like solution. Another merit of using Mf is that myosin in Mf is quite stable because of significant stabilization upon binding with actin. The state of myosin in Mf forming complex with actin is the same as in muscle after a complete consumption of ATP; rigor state. A very stable nature of actin is in the stabilizing effect. Thus, myosin denaturation in Mf or meat is referred as the one in a stabilized form by actin. Practically no detectable actin denaturation upon heating of fish Mf was reported [11]. The important finding was made on actin denaturation in Mf by Wakameda et al. [13, 14, 23]. Actin in Mf was selectively denatured by its treating with high concentrations of neutral salts such as 1.5-2 M KCl or NaCl. Actin denaturation consequently accelerated myosin denaturation by losing protective effect on myosin denaturation. High salt-induced actin denaturation was also reported with actomyosin dissolved in 0.6 M KCl upon its frozen storage at -10°C [7]. To prevent such actin denaturation, storage at -20°C or by reduction of KCl concentration to 0.1 M was recommended. By accepting the suggestion and as the practical frozen storage temperature of fish meat commonly used is around -20°C, myosin freeze denaturation study has been conducted at about -20°C by using meat itself or Mf suspended in 0.1 M KCl or NaCl. By using Mf, quantitative protective effect of organic compounds such as sugars, organic acid salts, amino acids on myosin denaturation were

studied [4, 24].

To distinguish myosin denaturation at head and tail portions, chymotryptic digestion method was proposed [11]. The principle of the method is to detect exposed hydrophobic amino acid residues as a consequence of structural changes of myosin molecule. The method is applicable to any proteins in the digestion medium. The method was successfully applied to myosin denaturation study in frozen stored kuruma prawn Mf. We found that actin in frozen stored prawn Mf was readily degraded and remaining actin content in the digest became faint even when Mf was stored at 0.1 M NaCl [15]. The result clearly demonstrated that a quick actin denaturation upon frozen storage of prawn Mf. Actin denaturation was similarly observed when flounder Mf suspended in 0.1 M NaCl was frozen stored [16]. The finding was unexpected because actin in Mf suspended in low-salt medium is believed to be stable [7]. We also found that myosin denaturation and actin denaturation progressed in parallel during the storage period. The most possible reason would be that denatured actin was no longer unable to protect myosin from denaturation and consequently myosin denaturation was accelerated. In other words, unstable flounder and kuruma prawn myosin denatured immediately after the loss of protection. To understand the role of actin denaturation in a quick denaturation of myosin in frozen Mf, tilapia Mf was used. We expected to detect myosin denaturation after detaching from denatured actin tilapia because tilapia myosin is one of the most stable fish myosin.

As a quick actin denaturation was observed with tilapia Mf upon its frozen storage at any temperatures from -10 to -40°C, actin denaturation would be commonly observable event among any Mf. Actin denaturation was not prevented by lowering storage temperature to

276 -40°C or by quick freezing in dry-ice acetone (data not shown). With tilapia Mf,
277 Ca^{2+}-ATPase inactivation and decrease of amount of S-1 production was much slower than
278 actin denaturation. When Mf was frozen stored at -40°C, Ca^{2+}-ATPase activity was kept
279 high for a long period of 160 days after actin denaturation occurring in a few days. In other
280 words, myosin denaturation observed with tilapia Mf would be myosin denaturation
281 without protection by actin. Probably, unstable flounder and kuruma prawn myosin
282 denatured immediately after detaching from denatured actin or actin denaturation
283 determined myosin denaturation.
284 Tilapia meat was frozen stored to study whether actin denaturation is detectable with meat
285 like Mf. Ca^{2+}-ATPase inactivation progressed very slowly compared with Mf showing that
286 myosin is kept stable in frozen stored meat. The slow myosin denaturation was explained
287 by no actin denaturation in frozen stored meat revealed by chymotryptic digestion.
288 Probably, actin kept preventing myosin from denaturation during frozen storage of meat.
289 The difference in the Ca^{2+}-ATPase inactivation rate between Mf and meat was about 9
290 times. The difference would be stabilizing effect of actin on myosin during frozen storage.
291 Inactivation rates at -20 and -40°C for Mf and for meat were too small to compare. So the
292 rates were compared only at -10°C.
293 Although there was a significant difference between frozen stored Mf and meat in actin
294 denaturation, we found similar changes in myosin denaturation. Very slow denaturation at
295 tail portion of myosin molecule for both samples was observed, which explained high
296 salt-solubility of myosin.
297 At present, we have no clear explanation for actin denaturation in Mf. Imagining freezing

of Mf suspension, water in medium would be the material for ice crystal formation. Mf in the medium would be compressed by a large quantity of ice crystal from outside. Such physical force may damage actin filament in Mf. Moreover, salt condensation might happen during the process. These would be the reason for actin denaturation. On the other hand, ice crystal may be formed within muscle tissue for meat. The rigid muscle structure seemed prevent the damage of Mf structure. Although the data were not presented, Mf can be prepared from frozen fish meat and the Mf prepared is indistinguishable from one from fresh muscle showing striation on the surface. We observed Mf structure after its frozen storage on microscopy. Mf did not show striation on the surface and was aggregated. Even though Mf is a convenient material for myosin denaturation study, Mf cannot be used as a model material for studying freeze denaturation of myosin and the results obtained with Mf has to be reconsidered.

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371

372

373 **Figure captions:**

374 Fig. 1 Ca^{2+}-ATPase inactivation upon frozen storage of tilapia Mf. Mf suspended in 0.1 M
375 NaCl, 20 mM Tris-HCl (pH 7.5) was frozen stored at -10 (circles), -20 (squares), and -40°C
376 (triangles). Remaining Ca^{2+}-ATPase activity was measured at 25°C in the reaction medium
377 of 0.5 M KCl, 25 mM Tris-maleate (pH 7.0), 5 mM $CaCl_2$, and 1 mM ATP.

378

379 Fig. 2 Myosin and actin denaturation upon frozen storage of Mf at -10°C. (a) Changes in
380 Ca^{2+}-ATPase activity (circles), salt-solubility in 0.5 M NaCl in the presence of 1 mM
381 Mg-ATP (squares), and monomeric myosin content as measured by ammonium sulfate
382 fractionation in the presence of 1 mM Mg-ATP (triangles). (b) Changes in the amount of
383 S-1 (circles) and rod (squares) produced from Mf by chymotryptic digestion and
384 chymotrysin registrant actin content in the digest (closed circles). The patterns in Fig. 3a
385 were used to measure.

386

387 Fig. 3 Changes in chymotryptic digestion pattern of Mf upon its frozen storage. Frozen
388 stored Mf at -10 (a), -20 (b), and -40°C (c) was digested with chymotrypsin (1/200,w/w,
389 20°C for 60 min) in the reaction medium of 0.05 M NaCl, 20 mM Tris-maleate (pH 7.0),
390 and 1 mM EDTA. MHC, Act, Rod, and S-1 denote myosin heavy chain, actin, myosin rod,
391 and myosin subfragment-1 bands, respectively. w denotes Mf before digestion.

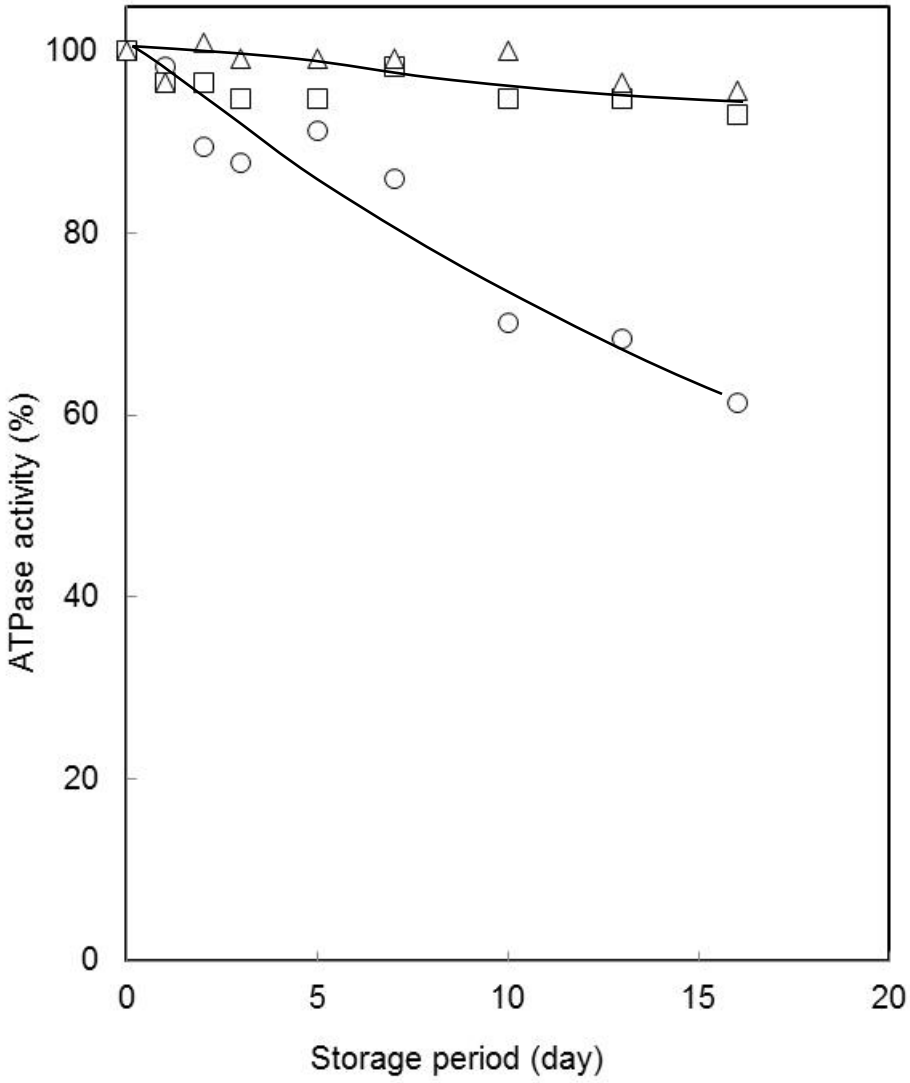
392

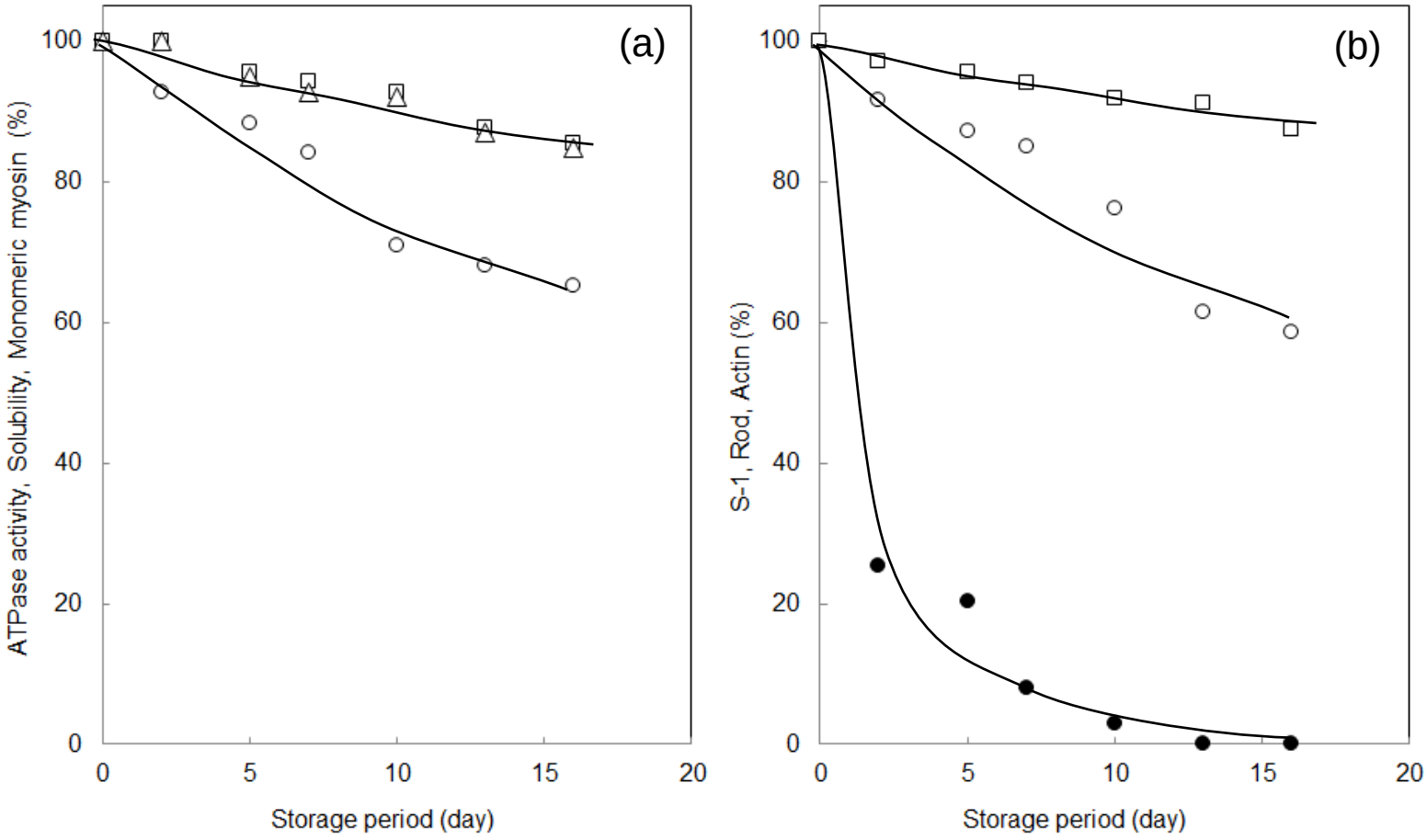
393 Fig. 4 Ca^{2+}-ATPase inactivation upon frozen storage of tilapia dorsal muscle. Tilapia meat
394 (~ 5 g) wrapped with plastic film was frozen stored at -10 (circles), -20 (squares), and

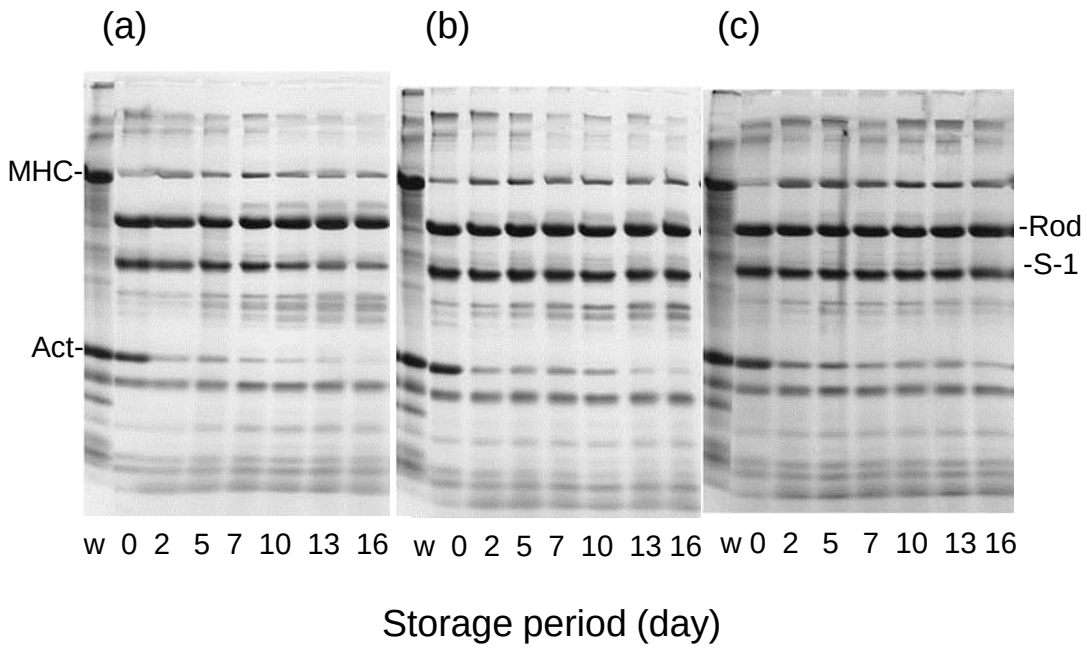
-40°C (triangles). Frozen meat was quantitatively converted into muscle homogenate, and remaining Ca^{2+}-ATPase activity was measured as in Fig. 1. Ca^{2+}-ATPase inactivation upon frozen storage of Mf (closed circles) was also shown.

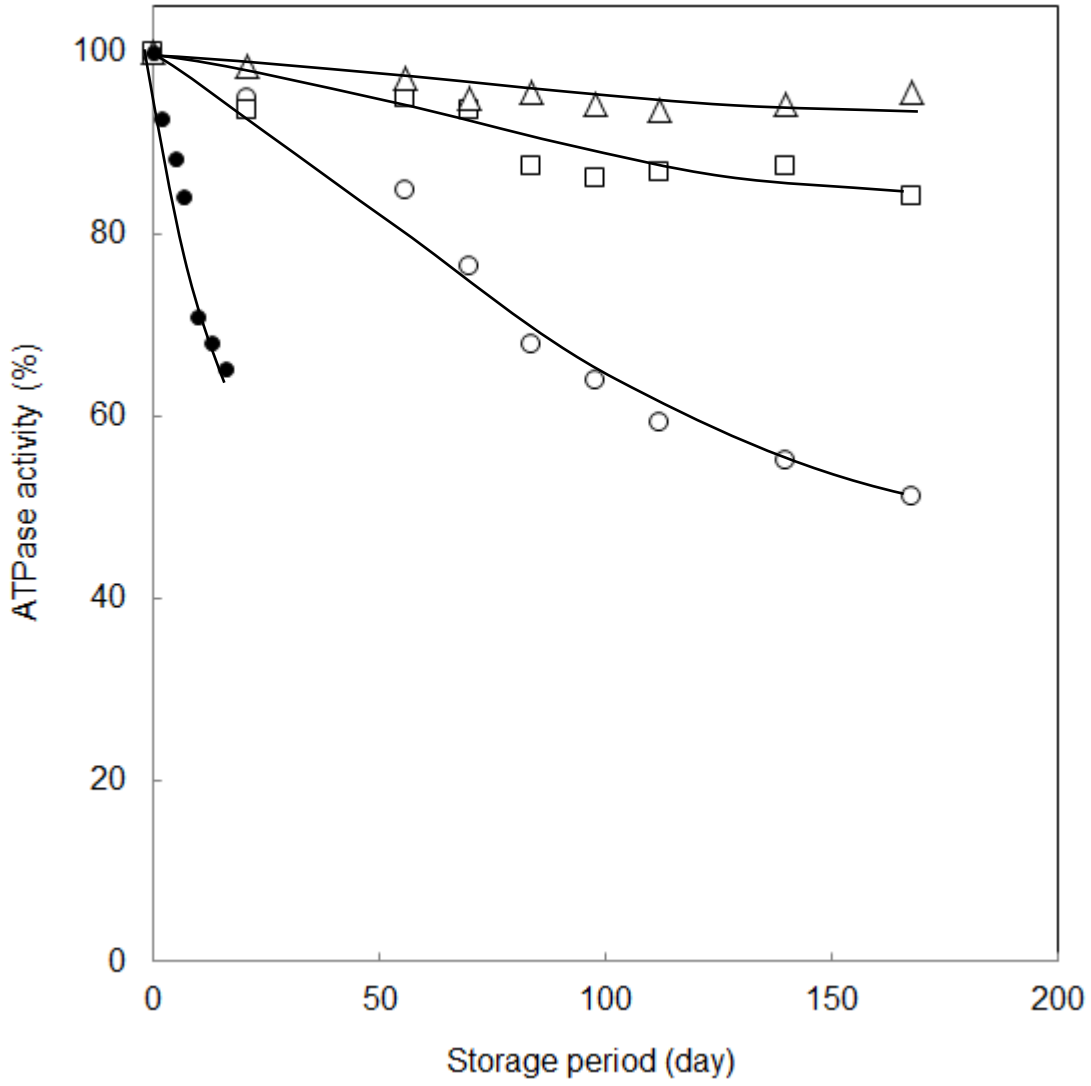
Fig. 5 Myosin and actin denaturation upon frozen storage of meat at -10°C. The same homogenates as in Fig. 4 were also used to study myosin and actin denaturation in frozen stored meat. (a) Changes in Ca^{2+}-ATPase activity (circles), salt-solubility in 0.5 M NaCl in the presence of 1 mM Mg-ATP (squares), and monomeric myosin content as measured by ammonium sulfate fractionation in the presence of 1 mM Mg-ATP (triangles). (b) Changes in the amount of S-1 (circles) and rod (squares) produced from muscle homogenate by chymotryptic digestion and amount actin content in the digest (closed circles). Other conditions were the same as in Fig. 2.

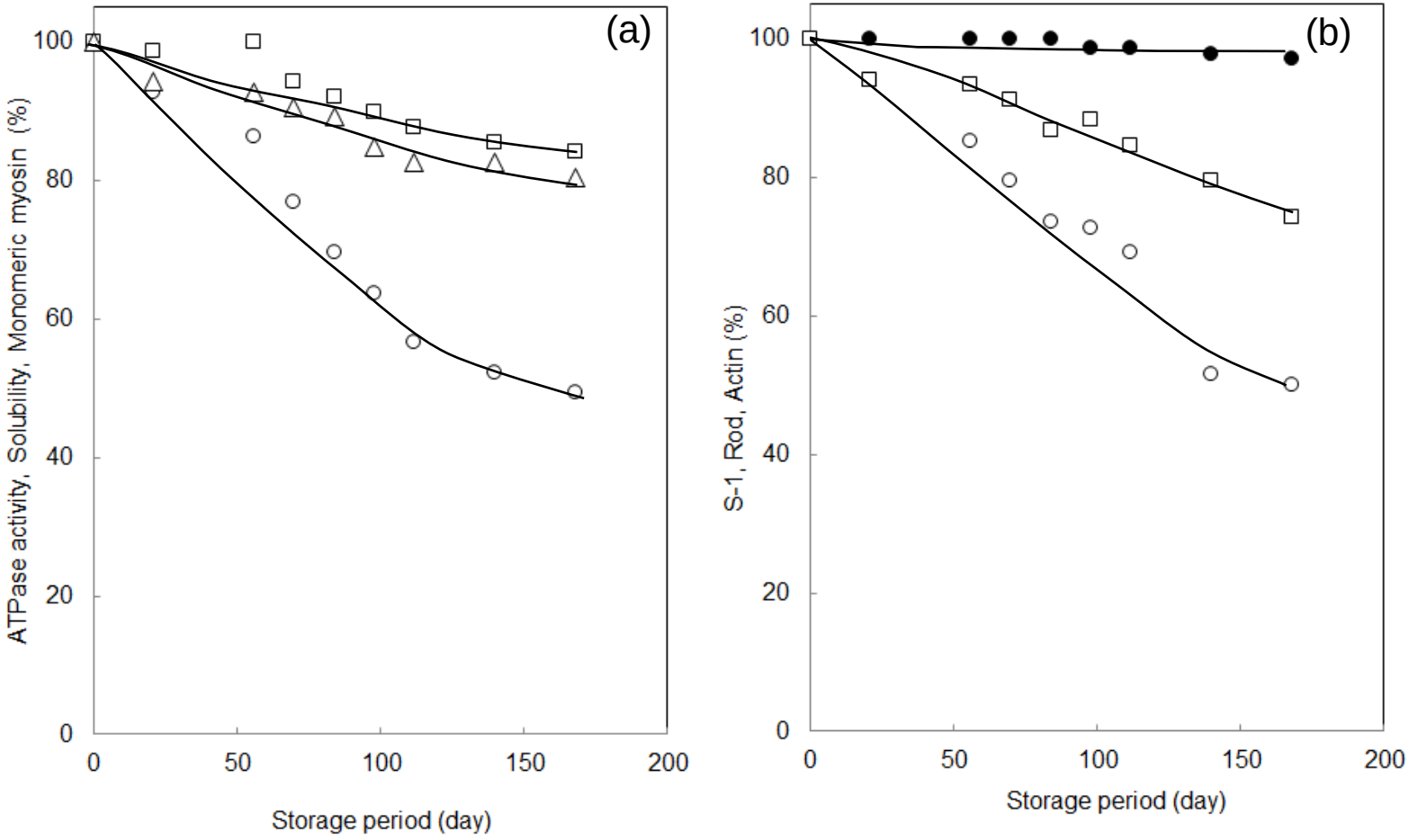
Fig. 6 Chymotryptic digestion patterns of muscle homogenate prepared from frozen stored meat. Muscle homogenates prepared from meat before freezing (a) and ones from frozen stored meat for 170 days at -10 (b), -20 (c), and -40°C (d) were digested as in Fig. 3. (w) and (d) denote homogenate itself and its digest, respectively. The same abbreviations as in Fig. 3 were also used.

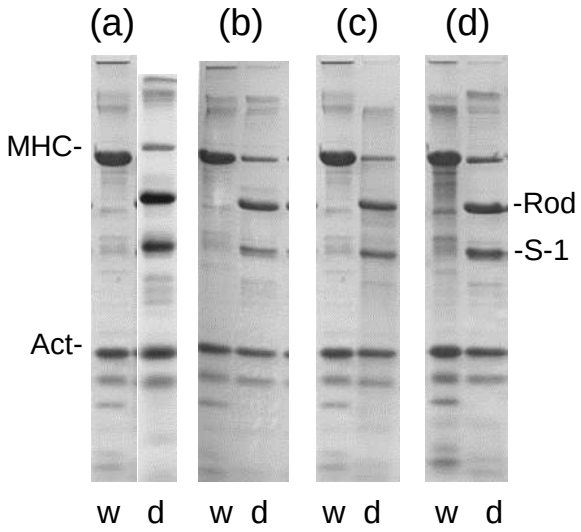

100
80
60
40
20
0
ATPase activity (%)
0
5
10
15
20
Storage period (day)


(a)
ATPase activity, Solubility, Monomeric myosin (%)
100
80
60
40
20
0
0
5
10
15
20
Storage period (day)
(b)
S-1, Rod, Actin (%)
100
80
60
40
20
0
0
5
10
15
20
Storage period (day)


(a)
(b)
(c)
MHC-
Act-
-Rod
-S-1
w 0 2 5 7 10 13 16
w 0 2 5 7 10 13 16
w 0 2 5 7 10 13 16
Storage period (day)


100
80
60
40
20
0
ATPase activity (%)
0
50
100
150
200
Storage period (day)


(a)
ATPase activity, Solubility, Monomeric myosin (%)
100
80
60
40
20
0
0
50
100
150
200
Storage period (day)
(b)
S-1, Rod, Actin (%)
100
80
60
40
20
0
0
50
100
150
200
Storage period (day)


(a)
(b)
(c)
(d)
MHC-
Act-
-Rod
-S-1
w
d
w
d
w
d
w
d