Below-zero storage of fish to suppress loss of freshness

Takeya Yoshioka¹ · Yoshiko Konno² · Kunihiro Konno²

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Abstract

The decomposition of ATP in flounder and greenling muscle were compared at 0 and -2°C . The decomposition of inosine-5-monophosphate (IMP) and subsequent increase in the K -value were suppressed at -2°C for both species, although the K -value increased much more slowly for flounder than for greenling. When flounder was stored at 0°C , a high IMP content was maintained for more than 10 days, and then dropped quickly. This quick reduction in the IMP content was not observed at -2°C . The fast reduction in the IMP content at 0°C was explained by the activity of an IMP-decomposing enzyme produced by spoilage microorganisms; it no longer occurred when the meat was stored in the presence of 150 p.p.m. of the antibiotic chloramphenicol. 5'-Nucleotidase produced by the bacteria was less stable than that produced endogenously. Spoilage bacteria also produced a strong protease that degraded muscle protein. It was concluded that lowering the storage temperature of flounder and greenling from 0 to -2°C suppressed the growth of spoilage bacteria and slowed the increase in the K -value.

Keywords Inosine-5-monophosphate · K -value · 5'-Nucleotidase · Super-chilling · Decomposition

Introduction

Freshness is a key factor that determines the quality of fish and other marine organisms for consumption. Many indices are used to assess freshness. Sensory indices include gill color, clearness of the eyeball, state of the scales, and ability to break fish at the abdomen, etc. The physical state of fish before or after rigor mortis is also an important factor used to judge freshness. An increase in the volatile nitrogen content caused by the growth of spoilage microorganisms is the most often used chemical index of freshness in fish. For fish consumed as sashimi, the K -value, which is a sensitive index of freshness, is used to assess quality (Arai and

Saito 1961; Saito et al. 1959; Uchiyama and Ehira 1970; Uchiyama and Kakuda 1984). This index is commonly used because it is related to the decomposition of ATP, which is associated with rigor mortis. Rigor mortis occurs when ATP is completely consumed in muscle. Live fish muscle contains roughly 10 mM ATP, and ATP is consumed mainly to provide energy for muscle contraction when fish swim. Once the oxygen supply stops as a result of death, ATP regeneration from ADP becomes negligible. At this stage a fish goes into rigor mortis. ADP is further decomposed into the much simpler compound, hypoxanthine (Hx), through a series of reactions. All of the processes from ATP to Hx in fish muscle are catalyzed by step-specific enzymes. The slowest step in the series is the one converting inosine-5-monophosphate (IMP) to inosine (HxR), which is catalyzed by 5'-nucleotidase (EC 3.1.3.5). Fortunately, compounds that contain no phosphate (HxR and Hx) can be easily separated from those that do, such as those in the conversion of ATP to IMP, by their different negative charge. The K -value is defined as the relative amount of HxR + Hx to the total amount of ATP-related compounds (Saito et al. 1959; Tejada 2009). As all metabolic processes are catalyzed by enzymes, and as enzymatic reactions are determined by temperature and time of storage, the K -value indicates the storage history of fish. The period needed to reach a K -value of 100% for many

✉ Takeya Yoshioka
yoshioka@techakodate.or.jp

Yoshiko Konno
konnodpu@163.com

Kunihiro Konno
konno@fish.hokudai.ac.jp

¹ Hokkaido Industrial Technology Center, Hakodate,
Hokkaido 041-0801, Japan

² Laboratory of Marine Food Science, Graduate
School of Fisheries, Hokkaido University, Hakodate,
Hokkaido 041-8611, Japan

fish species ranges from a few days to a few weeks, similar to the handling periods of raw fish.

Not only are the activities of enzymes involved in determining the *K*-value temperature and time dependent, but so are other enzyme reactions, such as degradation by protease. Therefore, storing fish at a lower temperature is considered the simplest method to suppress enzyme activity. Fish are usually distributed on ice, at 0 °C. Attempts have been made to maintain fish quality by storing fish below zero (Endo 1989), which is termed super-chilling storage (Ando et al. 2004; Gokoglu and Yerlikaya 2015). The storage of fish in a mixture of salt water and ice is the simplest method for this. Innovative attempts have been made to produce fine ice crystals from salt water, slurry ice or sherbet ice, which can be pumped and are soft, so do not damage the skin of fish during transportation.

In this study we examined whether the storage of fish meat below zero (at −2 °C) suppresses its loss of freshness. Two species of fish, greenling *Hexagrammos otakii* and flounder *Paralichthys olivaceus*, were used for this study because they have different *K*-value profiles. Greenling shows a quick increase in the *K*-value, whereas flounder shows a very slow increase. The effect of a lower storage temperature, to −2 °C, on the increase in the *K*-value was studied for these two species. It has been reported that the increase in the *K*-value of flounder is influenced by the growth of microorganism, with an abrupt increase in the *K*-value in the latter phase of storage (Wu et al. 2016). We examined whether lowering the storage temperature suppressed the growth of microorganisms. We also examined whether the IMP-decomposing enzyme produced by the microorganisms is the same as the endogenous one present in fish muscle.

Materials and methods

Fish

Live greenling and flounder were purchased at a local fish market. The fish were instantly killed by cutting the vein near the gill cover, then transported to the laboratory. The fish fillets were cut into small pieces (roughly 5 g) and wrapped with plastic film. The fillets were stored in a refrigerator at 0 or −2 °C (Twinbird SC-DF25); −2 °C was chosen to simulate super-chilling storage. Some experiments were conducted with minced muscle rather than chunks of muscle to mix muscle homogeneously with chloramphenicol (CP; 150 p.p.m.) to prevent bacterial growth. Meat was blended four times in a Panasonic MK-K48P for 30 s. No signs of freezing were detected for either type of film-wrapped sample throughout the storage period at −2 °C.

ATP degradation analysis

ATP and related compounds were extracted from 1 g of meat by using the simplified method developed by Hu et al. (2013). Briefly, 10 ml 5% perchloric acid was added to fish samples in 50-ml disposable centrifuge tubes and mixed well with a glass rod for 15 min on ice. To adjust the pH of the solution to around pH 3, we added 5.2 ml of 1 M KOH and 4.7 ml of H₂O. The supernatant was filtered through a 0.45-μm membrane filter (Tosoh W-13-2) on ice. The sample was analyzed on a Shodex Asahipack GS-320HQ using a mobile medium of 0.1 M phosphate buffer (pH 2.8) following Matsumoto and Yamanaka (1990). The *K*-value was calculated from the contents of ATP and related compounds in the sample following Wu et al. (2016).

Muscle homogenate preparation

5'-Nucleotidase catalyzes the phosphorolytic cleavage of IMP to HxR (Tomioka and Endo 1984; Nedachi and Hirota 1991). To analyze the activity of 5'-nucleotidase, minced muscle was homogenized four times in six volumes of 0.1 M NaCl and 20 mM Tris-HCl (pH 7.5) at 16,000 r.p.m. for 30 s. The homogenate was thoroughly dialyzed against the same solution and the dialysate used directly as the crude enzyme sample.

Measurement of 5'-nucleotidase activity

Nucleotidase activity was measured in a reaction medium of 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂ and 1 mM IMP at 25 °C following Tomioka and Endo (1984). Inorganic phosphate liberated from IMP was measured by a colorimetric method used for myosin ATPase measurement (Fiske and Subbarow 1925).

Thermal stability of endogenous and microbial 5'-nucleotidase

Flounder mince was stored at 0 °C for 16 days with or without 150 p.p.m. CP. The mince stored with CP was assumed to contain only endogenous 5'-nucleotidase, whereas the mince stored without CP was assumed to contain endogenous and microbial 5'-nucleotidase. Muscle homogenate in test tubes was incubated at various temperatures for 30 min, or incubated at 50 °C for various periods of time. The remaining 5'-nucleotidase activity was assayed to assess the thermal stability of the enzyme derived endogenously and microbially. The samples were also heated in the presence of various protease inhibitors to study the effect of this on the protease produced by the microorganisms. The inhibitors

were: phenylmethylsulfonyl fluoride (PMSF; 1 mM), trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64; 10 μ M), and 4 mM ethylenediaminetetraacetic acid (EDTA).

Production of proteolytic enzyme by microorganisms

Proteolytic degradation of muscular proteins by the protease produced by microorganisms was studied after storing muscle homogenate prepared from fresh flounder meat in a refrigerator at 0 °C for up to 16 days. The stored homogenate was mixed with an equal volume of 2% SDS, 8 M urea, 5% 2-mercaptoethanol, and 20 mM Tris–HCl (pH 7.5) to dissolve whole proteins. The dissolved-protein sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis following Laemmli (1970) using 7.5% polyacrylamide.

Results

Effect of lower storage temperature on IMP decomposition and *K*-value increase during greenling meat storage

A block of greenling muscle (roughly 5 g) wrapped with plastic film was stored at 0 (Fig. 1a) and –2 °C (Fig. 1b). Immediately after the instantaneous death of the fish, the muscle contained 6.8 μ mol/g ATP and a small amount of ADP (1.8 μ mol/g meat), which indicated that the fish died without struggling. ATP was no longer detectable after 1 day's storage at either temperature. ADP also decreased to its lowest level of ca. 0.5 μ mol/g after 1 day. As ADP was constantly observed throughout the storage period, this form

of ADP was considered to be different from the one produced by the hydrolysis of ATP in muscle, and thought to be that bound to F-actin, which binds 1 mole of ADP and Ca^{2+} for its stabilization (Ooi and Okagaki 2011). The ADP concentration detected was explained by the molar concentration of G-actin. A quick drop in ATP was accompanied by the generation of IMP at both temperatures. AMP was not detected, which indicated the fast conversion of AMP to IMP. The most prominent compound in muscle that had been stored for 1 day was IMP, with a small amount of Hx. There was a gradual decrease in the IMP content and increase in the Hx content with storage. IMP decomposition and Hx accumulation were faster at 0 °C than at –2 °C.

The *K*-values for the two samples stored at 0 and –2 °C were calculated and are presented in Fig. 2. It took 1.5 days for the *K*-value to reach 20% at 0 °C, while at –2 °C this took 4 days. Lowering the storage temperature from 0 to –2 °C clearly suppressed an increase in the *K*-value. The magnitude of the suppression was quite large.

Effect of reduced storage temperature on IMP decomposition in flounder meat

It has been reported that the decrease in the IMP content is biphasic for flounder, showing a very slow decrease and then a quick drop in the late phase (Wu et al. 2016). The growth of microorganisms is involved in the fast reduction of IMP content (Matsumoto and Yamanaka 1991; Surette et al. 1988). Taking this into consideration, flounder meat was stored with and without CP. To mix CP well with the meat, minced muscle was employed. Mincing of fish meat accelerated ATP decomposition; IMP was the major compound found in the minced meat. The IMP decomposition profile during storage was unaffected by mincing (Wu et al.

Fig. 1 Effect of storage temperature on the ATP decomposition profile of greenling. Block of greenling meat (5–10 g) wrapped with plastic film was stored at 0 °C (a) or –2 °C (b). ATP and its related compounds were extracted from the stored meat by using 5% perchloric acid, and the composition of the extract was analyzed by high performance liquid chromatography with an Asahipack GS-320HQ column. Filled circles ATP, squares ADP, open circles IMP, triangles hypoxanthine (Hx). Levels of other compounds were beyond the limit of detection

Fig. 2 *K*-value increase for greenling meat stored at 0 and -2°C . *K*-values calculated from data in Fig. 1. Increase in *K*-value at 0°C (circles) and -2°C (triangles)

2016). One mince sample containing no CP (Fig. 3a, c) and one containing 150 p.p.m. CP (Fig. 3b, d) were stored at 0°C (Fig. 3a, b) and -2°C (Fig. 3c, d), and ATP decomposition followed. The initial ATP content of the mince was low ($2.1\ \mu\text{mol/g}$ meat), and ADP was the major compound ($10.8\ \mu\text{mol/g}$ meat) produced, indicating that ATP was almost completely hydrolyzed to ADP during the mincing process. This quick conversion of ATP to ADP in the mince is explained by the hydrolysis of ATP to ADP, which is catalyzed mainly by Mg^{2+} -ATPase in myosin in muscle. The activity of the Mg^{2+} -ATPase is controlled by a change in Ca^{2+} concentration, namely, the regulation of muscle contraction by Ca^{2+} . When the muscle is in a relaxed state, the Ca^{2+} level is restored in the sarcoplasmic reticulum and consequently the activity of Mg^{2+} -ATPase is significantly suppressed (Ebashi et al. 1968). Blending the meat destroys the sarcoplasmic reticulum membrane and releases Ca^{2+} from it. The increased Ca^{2+} concentration accelerates the actin-activated Mg^{2+} -ATPase activity of myosin. The high ADP content initially observed decreased rapidly within 2 days, but a small amount of ADP was observed throughout the storage period of greenling. This ADP must have been previously bound to F-actin. When the meat was stored for longer than 2 days, the major compound observed was IMP. The decrease in IMP content was significantly slower in flounder than in greenling, as previously reported (Arai

and Saito 1961). A quick drop in the IMP content on ca. day 12 was characteristic for flounder stored at 0°C without CP (Fig. 3a), while this drop was not detected when the meat was stored with CP (Fig. 3b). The contribution of bacterial decomposition to the quick drop in the IMP content has been previously confirmed (Wu et al. 2016). On the other hand, when the mince was stored at -2°C , even in the absence of CP, such a quick drop in IMP content was not detected for up to 21 days (Fig. 3c). Lowering the storage temperature in the present study by 2° suppressed the growth of bacteria that caused the quick drop in the IMP content. The IMP content decreased slightly slower at -2°C with CP than without it (Fig. 3d).

K-values calculated from the data in Fig. 3 are shown in Fig. 4. The increase in the *K*-value for the sample stored at 0°C without CP was biphasic and with CP was monophasic (Fig. 4a). Both samples showed a similar increase in the *K*-value in the early phase, which corresponded to IMP decomposition by endogenous 5'-nucleotidase (Fig. 4a). These differences in *K*-value profiles with and without CP were not found at -2°C (Fig. 4b). The increase in the *K*-value was much slower at -2°C than at 0°C . At 0°C , a *K*-value of 20% was reached at 11 days, while at -2°C this occurred at 17 days. Lowering the storage temperature of flounder muscle was found to have two benefits: the suppression of an increase in the *K*-value, and the suppression of bacterial growth.

Characterization of 5'-nucleotidase produced by microorganisms in fish meat

Bacteria growing in flounder meat are thought to produce 5'-nucleotidase to decompose IMP. To confirm this, muscle homogenate prepared from fresh flounder was stored at 0°C (Fig. 5a) and -2°C (Fig. 5b) with and without CP. A gradual decrease in the activity of 5'-nucleotidase was found in the sample stored with CP (Fig. 5a, b), which was slightly faster at 0°C than at -2°C . This decrease would have been due to the very slow denaturation of the endogenous 5'-nucleotidase originally present in the flounder muscle. When the sample was stored at 0°C without CP, the activity of 5'-nucleotidase decreased slowly for up to 7 days, which was similar to that seen in the presence of CP, while a remarkable increase in activity followed. 5'-Nucleotidase increased 180% in activity over 19 days. When the homogenate was stored with CP at -2°C , there was a delayed increase in 5'-nucleotidase activity; the enzyme activity profile was similar to that without CP for up to 15 days, but then there was a small relative increase by day 19. This was basically the same as what was seen in stored flounder meat, namely a quick drop in the IMP content in the later phase of storage. The increase in activity in the homogenate (Fig. 5a) and in muscle (Fig. 3a) occurred over different periods. We

Fig. 3a–d Effect of storage temperature and chloramphenicol on the decomposition of inosine-5-monophosphate (IMP) in flounder mince. Minced flounder muscle (5 g) (a, c) with 150 p.p.m. chloramphenicol (CP) (b, d) wrapped with plastic film and stored either at 0 °C (a, b) or –2 °C (c, d). Filled circles ATP, squares ADP, open circles IMP, Hx triangles

conclude that lowering the storage temperature by 2° C must have suppressed bacterial growth in the muscle homogenate.

It is interesting to know whether the 5'-nucleotidase produced by the bacteria is the same form as that produced endogenously. To answer this, samples were prepared containing endogenous enzyme alone and both endogenous and bacterial enzyme, as it is only possible to measure the bacterial enzyme alone by isolation of the bacteria. Based on the results in Fig. 3a, b, flounder mince was stored at 0 °C for 16 days with or without CP. The mince stored without CP must have contained the enzyme from both sources, while the mince with CP would have contained only the endogenous enzyme. Bacterial growth in the sample without CP was determined by the production of a spoilage smell. No such smell was detected in the sample with CP. Homogenates were prepared from these two

stored flounder minces. IMP decomposition in the mince homogenate without CP was 220% higher than that in the meat containing CP (Fig. 6). A difference was detected in the thermal stability of the endogenous and bacterial enzyme. The homogenates were heated for 30 min at various temperatures up to 65 °C and the remaining enzyme activities were plotted against temperature (Fig. 6). The activity of the endogenous enzyme decreased gradually as the temperature rose, with a complete loss of activity at 65 °C. In contrast, activity in the homogenate containing endogenous and bacterial enzymes showed a two-step decrease. The decrease at the low temperature range between 30 and 45 °C was significant, whereas very little inactivation was detected for the endogenous enzyme in this range. The second step was between 50 and 65 °C, a similar range to that for the endogenous enzyme

Fig. 4 Effect of storage temperature and chloramphenicol on the *K*-value of flounder mince. Data in Fig. 3 were used to calculate *K*-values under four conditions. *K*-value increase at 0 °C (a) and –2 °C (b) with (+CP; closed symbols) and without (–CP; open symbols) 150 p.p.m. CP

Fig. 5a, b Changes in 5'-nucleotidase activity during the storage of flounder muscle homogenate. Minced flounder muscle was homogenized with 0.1 M NaCl and 20 mM Tris–HCl (pH 7.5) and stored at 0 °C (a) or –2 °C (b) with (closed symbols) or without 150 p.p.m. CP (open symbols). 5'-Nucleotidase activity was measured in a reaction medium with 0.1 M NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM MgCl₂, and 1 mM IMP. Liberated inorganic phosphate was measured for the activity analysis

inactivation. The results clearly demonstrated that the enzyme produced by the bacteria was much less stable than the one produced endogenously. If a sample contains a mixture of the two types of enzyme with different stabilities, the remaining activity after complete inactivation of the less stable enzyme should be the same as that of the sample containing the more stable enzyme alone. The remaining activity at 45 and 50 °C for the decomposed mince (with bacteria) was roughly half that of the mince homogenate with CP, which suppressed bacterial growth. Thus, it is suggested that a second factor was responsible for the further decrease in the enzyme activity in the decomposed mince homogenate.

Contribution of protease to the thermal inactivation profile of bacterial 5'-nucleotidase

Thermal inactivation profiles for the muscle homogenates with and without CP were compared at 50 °C. If a sample contains two forms of the same enzyme with different stabilities, quick inactivation of the unstable form should be followed by slow inactivation of the stable form, shown by a break point in the activity profile of the enzyme. Moreover, the slope for either the fast or slow phase should be the same as the one for the sample containing a single form of the enzyme. The activity profile of homogenate containing only the endogenous enzyme gave a straight line when

Fig. 6 Thermal stabilities of endogenous and bacterial 5'-nucleotidase. Muscle homogenate was prepared as in Fig. 5 from flounder muscle mince without CP (open symbols) or with CP (closed symbols), which had been stored for 16 days. Homogenates were dialyzed against homogenizing buffer and heated in small test tubes for 30 min at various temperatures up to 65 °C. Activity was measured as in Fig. 5 and the remaining activity upon heating was calculated

analyzed by assuming a first-order reaction (Fig. 7a, closed symbols). The profile of the homogenate from decomposed mince that contained the two types of enzyme was complicated. As the profile showed a breaking point, it was certain that the homogenate contained enzymes with different

stabilities (Fig. 7a, open symbols). A very quick inactivation in the early phase indicated the presence of a very unstable form of the enzyme, which was well supported by the results in Fig. 6, which indicated inactivation at a low temperature range. The slope for the second inactivation phase was steeper than that for the endogenous enzyme. If a sample contains two types of enzyme with different stabilities, the second slope should be the same as that for the stable enzyme. A much steeper slope in the latter phase of inactivation than that for a single form of the enzyme again indicated the presence of a second form. After conducting some further experiments, the contribution of protease to this phenomenon was suggested. The homogenate containing two forms of the enzyme was incubated together with several types of protease inhibitors and the subsequent inactivation profiles studied (Fig. 7b). The addition of E64 did not change the profile at all; the addition of EDTA actually promoted the inactivation in both phases; and the addition of PMSF produced a slope similar to that for the endogenous enzyme (Fig. 7b, triangles). PMSF must have inhibited the protease that degraded the endogenous 5'-nucleotidase. However, the extent of inactivation achieved in the early phase was much larger than that achieved in the early phase for the mixture of the two types of 5'-nucleotidase. These data suggest that multiple proteolytic enzymes are involved in these events. As EDTA addition accelerated the inactivation at both phases, the effect of proteinase was not as simple as first thought.

Production of proteinase by bacteria

Bacteria growing on the muscle produced protease together with 5'-nucleotidase. The production of protease was

Fig. 7 Thermal inactivation profiles of endogenous and bacterial 5'-nucleotidase and effect of protease inhibitor on their profiles. Muscle homogenates used in Fig. 6 were heated to 50 °C for various periods and remaining activity was analyzed by using a first-order reaction (a). Closed symbols +CP, open symbol -CP. Heating was conducted in the presence of various types of protease inhibitors, and enzyme activities similarly analyzed (b). Inhibitors: 4 mM ethylenediaminetetraacetic acid (EDTA; diamonds), 20 μ M trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64; squares), and 1 mM phenylmethylsulfonyl fluoride (PMSF; triangles)

confirmed by observing the degradation of muscular proteins during the storage of the muscle homogenate at 0 °C. Muscle homogenate from fresh meat was stored at 0 °C with and without CP. Changes in the SDS-PAGE of the muscle homogenate due to proteolytic degradation of muscle protein by protease was followed (Fig. 8). Even though the homogenate contained no CP, there was no change in the pattern of proteolytic degradation of muscle protein up to 14 days, while a significant degradation of myosin, actin, and other components was obvious when muscle protein was stored for 17 and 21 days (Fig. 8a). Myosin and actin were almost completely degraded by day 21. This degradation occurred between days 17 and 21, when the proteolytic enzyme responsible for this process seemed to be very active (Fig. 8a). However, practically no change in the pattern was observed when the meat was stored with CP. As CP is not thought to inhibit proteolytic enzymes, this indicated that endogenous proteolytic activity in flounder meat was negligible. As the IMP decomposing activity for the homogenate stored at 0 °C for 21 days without CP addition was quite high (Fig. 4a), the degradation of the bacterial IMPase by a bacterial protease seemed unlikely.

Discussion

It is well known that *K*-value profiles in fish are species specific, although the reasons for this are not clear (Tsuchimoto et al. 1988). The *K*-value in greenling increased

quickly, reaching 70% in a week when the meat was stored at 0 °C (Fig. 2). In contrast, the *K*-value of flounder increased very slowly, and after 1 week of storage it was still below 15% (Fig. 4). The *K*-value increase for greenling was monophasic, but that for flounder biphasic, as also reported in Wu et al. (2016). The second rapid increase in the *K*-value in flounder was due to the growth of microorganisms; this was supported by suppression of this quick increase by the addition of the antibiotic chloramphenicol.

The suppressive effect of lowering the storage temperature from 0 to −2 °C was studied in these two species of fish. The freezing of meat at the temperatures used was a concern, but there was no sign of this throughout the storage period of 3 weeks when the fish meat was wrapped with plastic film. Lowering the storage temperature to −2 °C clearly suppressed the *K*-value increase for both fish species. The *K*-value for greenling stored for 7 days at 0 °C was 72% and for flounder stored at −2 °C was 37%. When the temperature was decreased to −2 °C the storage period needed to attain a *K*-value of 30% lengthened from 2.5 to 5.5 days, i.e., it roughly doubled. Similarly, the storage of flounder at −2 °C suppressed an increase in the *K*-value; the increase was very slow, and the time needed to reach a *K*-value of 20% was extended from 11 to 17 days. A storage temperature of −2 °C almost doubled the potential storage period. This was expected because the activity of the IMP-decomposing enzyme, 5'-nucleotidase, is suppressed at lower temperatures. Meat can be much more successfully stored unfrozen at a temperature lower than −2 °C. However, the process of freezing usually damages the quality of fish, thus determining a suitable temperature for the storage of target fish species is important. Wrapping fish meat to prevent direct contact with a cold storage medium may also be helpful.

When *K*-values increase more quickly than that seen in flounder, the role of spoilage microorganisms has to be considered (Wu et al. 2016). A sudden increase in the *K*-value on day 15 was characteristic in flounder, where the *K*-value increased from 21 to 94% in a period of only 3 days (Fig. 3). The *K*-values on days 6 and 12 were 13 and 17%, respectively, i.e., showing only a 4% increase in 6 days. Thus, it was concluded that the activity of 5'-nucleotidase produced by spoilage bacteria was very high. When CP was added to the mince at 150 p.p.m., the *K*-value increased slowly throughout the storage period. However, the use of antibiotics to prevent the growth of microorganisms in food is not permitted. Thus, lowering the storage temperature to −2 °C is a simple and useful technique for the suppression of bacterial growth in fish for consumption (Fig. 4b), as an abrupt increase in the *K*-value was not detected at this temperature for up to 21 days. Employing a storage temperature of −2 °C has two merits: it reduces enzyme activity, which extends the time required to achieve the same *K*-value as in fish stored at 0 °C; it suppresses the growth of spoilage bacteria.

Fig. 8 Degradation of myofibrillar proteins during the storage of flounder muscle homogenate. The same homogenate as in Fig. 4 was used. The homogenates were stored at 0 °C in the absence (a) and presence (b) of 150 p.p.m. CP. The protein composition was analyzed by 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% SDS. The homogenate was fully dissolved by adding 8 M urea, 2% SDS, and 5% 2-mercaptoethanol and shaking overnight at room temperature. *MHC* Myosin heavy chain

The growth of spoilage bacteria that produce 5'-nucleotidase was confirmed not only in mince but also in muscle homogenate (Figs. 3, 5), and the suppressive effect of lowering the storage temperature by 2° C on the growth of microorganisms was also confirmed (Figs. 3, 5).

The 5'-nucleotidase produced by the bacteria was distinguished from the endogenously produced enzyme by its thermal stability. The bacterial enzyme was much less stable than the endogenous one. Heating at 40 °C for 30 min was sufficient to inactivate the bacterial enzyme, while the endogenous one required a much higher temperature of 65 °C (Fig. 6). Even though bacterial 5'-nucleotidase activity is very high, its stability is very low. Spoilage bacteria grown in the flounder meat produced a strong proteolytic enzyme that inactivated the endogenous 5'-nucleotidase during heating. An inhibition test suggested that serine protease was the enzyme involved in this (Fig. 7). The production of a strongly proteolytic enzyme by the spoilage bacteria was confirmed by analyzing myofibrillar protein degradation in stored muscle homogenate. Homogenate stored for up to 14 days at 0 °C showed no degradation, thus activity of the endogenous proteolytic enzyme was considered negligible. Severe degradation of myosin and actin occurred on day 17, and they were almost completely degraded after 21 days. As no degradation of any myofibrillar proteins was observed when fish meat was stored with CP, endogenous protease in flounder meat was considered to be negligible and the protease responsible for the above was believed to have been produced by microorganisms.

In this study, we did not identify the microorganisms that contribute to the decomposition of IMP and the proteolytic degradation of myofibrillar proteins. It is very probable that different fish species, different fishing grounds, as well as storage conditions, affect the species of microorganisms and their growth in fish meat. For example, the microorganisms responsible for IMP decomposition in horse mackerel muscle were identified as *Pseudomonas fragi* and either *Pseudomonas veronii* or *Pseudomonas extremaustralis* (Seki and Hamada-Sato 2014). Further study is needed for the identification of the microorganisms active in fish meat degradation.

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