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Effect of Super-chilling Storage on Maintenance of Quality and Freshness of Swordtip Squid *Loligo Edulis*

Mami ANDO[1], Emi TAKENAGA[1], Soyomi HAMASE[1] and Akihiko YAMANE[2]

[1] *Faculty of Human Life Science, Department of Human Nutrition, Yamaguchi Prefectural University, Yamaguchi 753–8502, Japan*
[2] *Hyo-On* Lab. Inc., Tottori 683–0101, Japan

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The quality of squid deteriorates very rapidly after catch. To maintain the quality of squid for a longer period, we carried out super-chilling storage. Live swordtip squids *Loligo edulis* were killed and stored at −2℃ (super-chilling) and 5℃ (refrigeration). At selected time intervals, their external color, texture, and K-values were evaluated. Additionally, the levels of free amino acid, water-soluble and water-insoluble proteins, and viable cell counts were determined. In the samples stored using the super-chilling method, whitening of the exterior, elevation of K-values, and the increase of viable cell counts were suppressed. Regarding the meat structure, free amino acids, texture, and content of water-soluble and water-insoluble proteins, no differences were observed between the two different storage temperatures. These results suggest that super-chilling storage can prolong the period for which squid can be preserved and maintains a higher quality than refrigeration.

Keywords: squid, super-chilling, refrigeration, freshness, K-value, appearance, meat

Introduction

Squid is one of the most popular kinds of seafood in Japan and the squid catch in Japan reached 200,000 tons in 2003 (Ministry of Agriculture, Forestry and Fisheries of Japan, 2004). However, with the large volume of squid meat extract, bacteria can breed readily resulting in the loss of freshness (Shimizu and Takeda, 1952). Thus, shipping in refrigeration would result in lowered commercial value due to loss of freshness. In contrast, transportation in frozen storage is very effective for maintaining freshness. However, free drip occurs during thawing and thereby the *umami* taste is lost (Kohno, 1996). In addition, ice crystals damage muscle cells with consequent muscle softening (Shirai and Yoshiakawa, 1999; Ando and Miyoshi, 2002). These unsolved problems, which occur during frozen storage or refrigeration, have lead to the new "super-chilling" storage concept, which possesses the advantages of both freezing and refrigeration and has been receiving attention (Yamane, 1982).

Super-chilling refers to the temperature zone below 0℃, but where ice crystals are not generated. This is a different concept from "chilled" or "partial freezing." The super-chilling temperature zone, suitable for both fish and shellfish, is considered to be in the range between 0℃ and −2℃ (Yamane, 1982, 1996). When foods are stored at super-chilling temperatures, their respiratory metabolism is repressed and the aging process is slowed, with the maintenance of cell activity (Suzuki and Murata, 1999). Furthermore, when subjected to low temperature stress below 0℃, the cells increase their free amino acid and sugar contents in order to resist freezing (Bohnert and Jensen, 1996). In addition, maintaining the lower temperature results in the inhibition of the growth of harmful microorganisms (Yamane, 1986). Basic studies have been conducted on super-chilling, not only in the area of food storage, but also in the medical field, in the storage and transportation of organs (Shiba *et al.,* 1998). Thus, further development of the concept of super-chilling temperature is anticipated.

Several studies have been published on the super-chilling storage of perishable fish and seafood (Fukuma *et al.,* 1998, Ayaki *et al,* 1999, Ando *et al.,* 2002, Ando, *et al.,* 2004); however, such research is still in its early stage and many unsolved problems remain.

In the present study, in order to elucidate the effect of super-chilling storage on the preservation of freshness, swordtip squid *Loligo edulis* were used in a comparison study of the determining factors affecting meat quality after refrigeration or super-chilling storage.

Materials and Methods

Storage conditions and preparation of samples Live swordtip squids *Loligo edulis* were purchased from a city market. They were killed at the market by piercing them between their eyes in order to prevent any stress during transportation. The squids were packed in a cool box (approximately 5℃) and carried to our laboratory within 1 h after killing. Their heads and organs were removed and their length and weight (mean length: 15.6± 2.0 cm, mean weight: 159.9±17.1 g) were measured. In

E-mail: ando@yamaguchi-pu.ac.jp

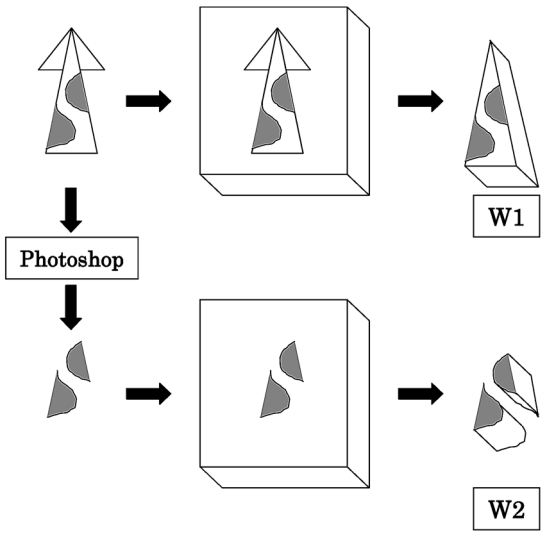

Fig. 1. Method for measuring the proportion of reddish regions on squid bodies.

the samples selected for the evaluation of external appearance, heads and organs were not removed because the removal procedure would damage the surface of the squids. Samples were stored for two days at −2℃ (super-chilling) and 5℃ (refrigeration) respectively. At each elected time, 12–16 individuals were used for each experiment. Super-chilling storage was carried out using an incubator (NH-60S, Ninomiya Sangyo Co., Tokyo, Japan). The incubator does not incorporate a cooling fan for maintaining an even temperature in the box and uses a Peltier device rather than a compressor for cooling, which avoids the effect of vibration interference.

Evaluation of change in external appearance The change in external appearance was evaluated as shown in Fig. 1. The external appearance of a squid body was photographed with a digital camera (FinePix500, Fujifilm Co., Tokyo, Japan) using automatic exposure in a darkroom. The picture was printed on photo paper and the squid body was cut out. The weight of the cut paper was measured (W1). Next, using image-editing software (Photoshop 5.0, Adobe, USA), reddish regions, including red, yellow, and black, were highlighted by selecting the maximum brightness and contrast for the original picture. The processed picture was then printed on photo paper. The reddish region was cut out and its weight (W2) was measured. The ratio of visible external reddish regions was evaluated using the following formula.

$$\text{Ratio of reddish region} = (W2/W1) \times 100(\%)$$

Measurement of physical strength Physical strength of the meat of 12–16 individuals was measured at room temperature (25℃) using a creepmeter (RE2-3305S, Yamaden Co., Tokyo, Japan) equipped with a cylindrical plunger (3 mm in diameter). The plunger pierced the meat from the inner side in a direction vertical to the muscle fibers at a speed of 1 mm/sec. Initially, the thickness of the meat was measured by the creepmeter, and the strength at 30% and 80% deformation of meat thickness was measured as the physical strength of the meat.

Histological observation Small blocks (3×3×10 mm) were cut from the meat and fixed in 5% glutaraldehyde (0.1 M phosphate buffer, pH 7.2). After dehydration with 50–100% ethanol, the blocks were embedded in resin (Technovit 7100, Kulzer Co., Germany). Thin sections (2 μm thick) were prepared by microtome and stained with 0.2% toluidine blue prior to observation under a light microscope (BX-50, Olympus, Tokyo, Japan).

K-values Meat extract was obtained by 10% perchlolic acid extraction. ATP related components were determined by HPLC, and K-values in the content of the meat extract were measured (Ando *et al.,* 2004).

Free amino acids Free amino acids in the meat extract were determined using an amino acid analyzer (L-8500, Hitachi, Tokyo, Japan).

General viable cell count One milliliter of sample solution, prepared by washing 2 g of meat with 10 mL of 0.9% NaCl, was smeared on an agar medium (Standard method agar, Nissui Pharm. Co., Tokyo, Japan) and plate cultured for 48 h at 37℃. The colonies were then counted to calculate the viable cell count per 1g of meat.

Determination of water-soluble and water-insoluble proteins Three grams of meat was homogenized (15000 rpm, 1 min) with 10 volumes of cold water and centrifuged at 10000 g for 15 min. Protein concentration in the supernatant (water-soluble protein) was determined according to the method of Lowry *et al.,* (1951). Protein concentration in the residue (water-insoluble protein) was determined by a micro-Kjeldahl method using 6.25 as the converting factor.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) Analysis of the protein components was carried out according to the method of Laemmli (1970) using 10% polyacrylamide gel as previously reported (Ando *et al.,* 2004). The supernatant obtained as described above (300 μl) was mixed with 300 μl of SDS-preparation buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 6% β- mercaptoethanol, pH 6.8) and heated in boiling water for 5 minutes. The water-insoluble fraction (approximately 30 mg) was put in the SDS-sample buffer (300 μl) and heated in boiling water for 5 minutes. Electrophoresis was performed at 20 mA and the 10% resolution gel was stained with Coomassie brilliant blue R-250 and destained with 7% acetic acid.

Results and Discussion

Changes in squid appearance The appearance of squids stored for 3 h and 12 h are shown in Fig. 2. The appearance at 3 h storage was that at the beginning of experiment in the laboratory. The 3 h include 1 h for transportation at 5℃ and 2 h for storage at each temperature. After 12 h storage, the sample stored in refrigeration exhibited a whitish appearance because of transparency loss. In contrast, samples stored according to the super-chilling method exhibited a reddish color. A reddish color is preferable for squid and maintaining such a color is important for the commercial value of the squid. Figure 3 shows the change in the ratio of the reddish region during storage. In the refrigerated sample, no

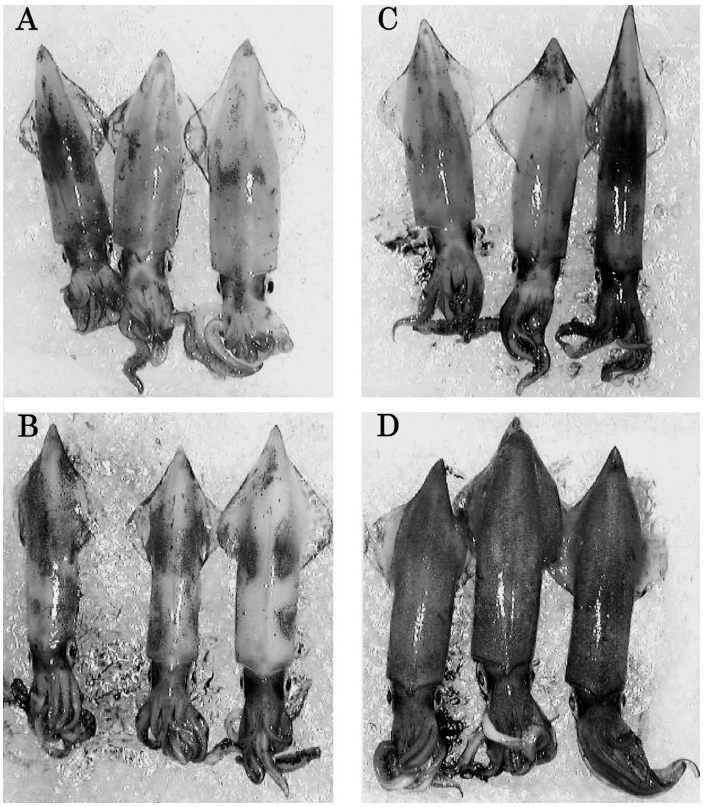

Fig. 2. External appearance of squid body. (A): refrigeration −3 h storage, (B): refrigeration −12 h storage, (C): super-chilling −3 h storage, (D): super-chilling −12 h storage.

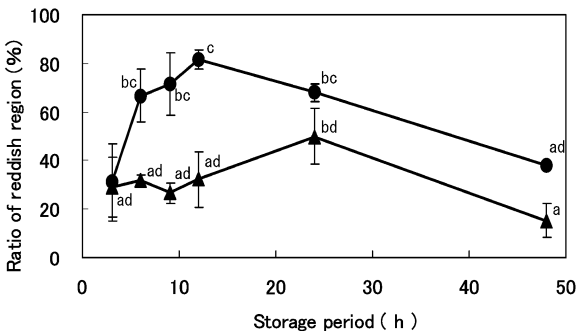

Fig. 3. Change in the ratio of colored regions on squid bodies. Each value is the mean of six individuals±SE. [abcd]Different letters with each set of data indicate significant differences ($p<0.05$). ▲: refrigeration, ●: super-chilling.

significant change in the reddish area was observed during the storage period except for at 24 h. However, in the super-chilling stored sample, the reddish color was maintained from 6 h to 24 h. This revealed that the super-chilling temperature could maintain the reddish color longer than the refrigeration temperature.

Yoshioka *et al.,* (2003) succeeded in maintaining the black color of Japanese common squid in storage at 0℃. The surface color of squids is generated by the expansion of chromatophore (Cloney and Florey, 1968). In general, chromatophore shrinks more at lower temperatures (Lin *et al.,* 1998). This shrinkage causes whitening of the squid body. When super-chilling squid, despite storing the squid at a lower temperature than during refrigeration, the reddish color of squid can be maintained longer. Although the mechanism of this phenomenon is unclear, at the super-chilling temperature the shrinkage of chromatophore may be inhibited in comparison with the refrigeration temperature.

Changes in physical properties and meat structure The changes in the physical properties of meat at deformation ratios of 30% and 80% are shown in Figs. 4 and 5. The value of the load denoting the surface hardness for a deformation ratio of 30% increased from the start to 9 h storage (Fig. 4). However, no differences were observed between the values for the two storage methods during 48 h storage.

The load required to produce a deformation ratio of 80% is equivalent to the force of gnaw off (Fig. 5). The experimental results revealed that, during both refrigeration and super-chilling, the meat softened for up to 9 h from capture as previously reported (Ando *et al.,* 1999; Kagawa *et al.,* 2002), and no significant difference in values was observed between the two storage methods.

In relation to these textural properties, there were no differences in the meat structure between the two storage methods (data not shown).

In our previous study, the softening of prawn meat during storage was inhibited by super-chilling storage (Ando *et al.,* 2004). The inhibition was due to a delay in the weakening of connective tissue bonding muscle fibers. However, it remains unclear why no such effect was observed for squid meat texture in the present study.

There are several protease activities in squid meat and they can degrade structural proteins (Sakai and Matsumoto, 1981). If the activities could be inhibited, the softening of meat might be suppressed. There is a possibility that the controlled temperature adopted in this study (−2℃) might not be sufficiently low to inhibit the activity. In addition, the low temperature used in the present study could not inhibit the biological reactions and reduce the change in pH. Therefore, a lower temperature may be required in order to produce the same effect as observed

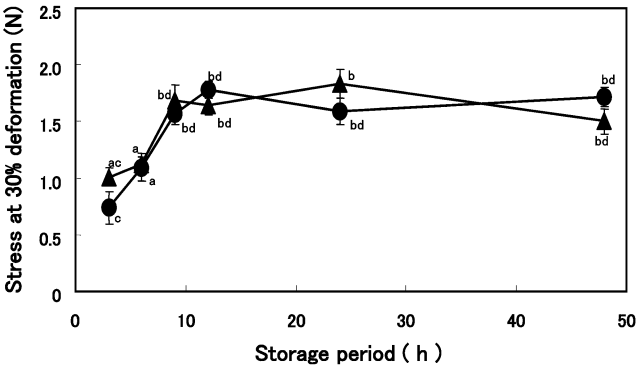

Fig. 4. Change in physical strength at 30% deformation of squid meat thickness during storage. Each value is the mean of six individuals±SE. [abcd]Different letters with each set of data indicate significant differences ($p<0.05$). ▲: refrigeration, ●: super-chilling.

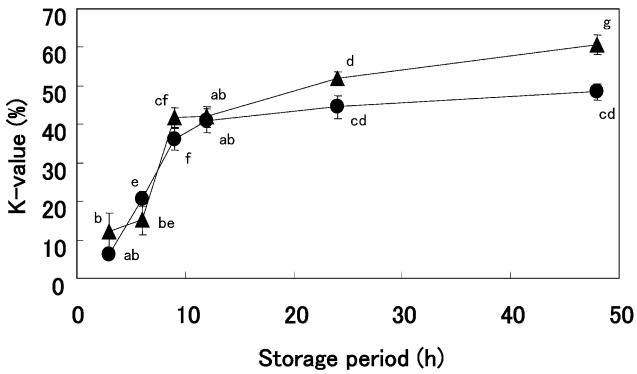

Fig. 6. Changes in K-values of meat during storage. Each value is the mean of six individuals±SE. [abcdefg]Different letters with each set of data indicate significant differences ($p<0.05$). ▲: refrigeration, ●: super-chilling.

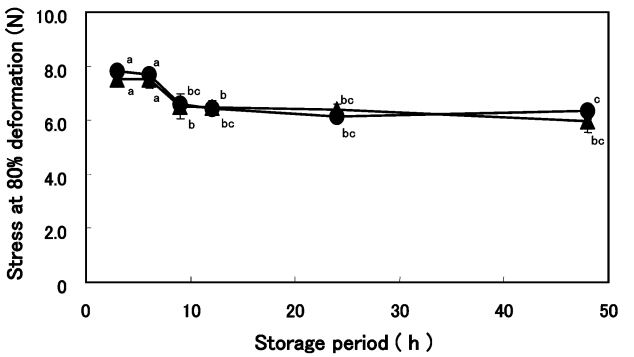

Fig. 5. Change in physical strength at 80% deformation of squid meat thickness during storage. Each value is the mean of six individuals±SE. [abc]Different letters with each set of data indicate significant differences ($p<0.05$). ▲: refrigeration, ●: super-chilling.

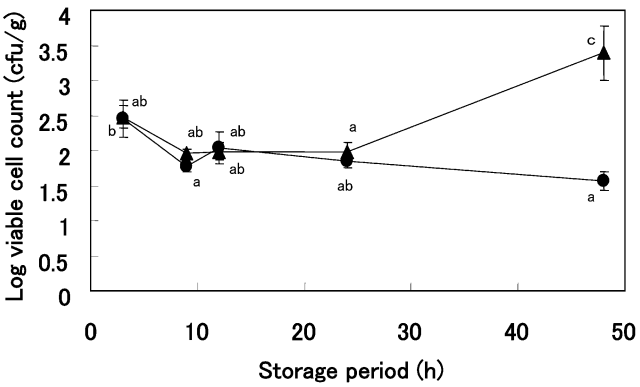

Fig. 7. Changes in viable cell counts. Each value is the mean of six individuals±SE. [abc]Different letters with each set of data indicate significant differences ($p<0.05$). ▲: refrigeration, ●: super-chilling.

Table 1. Protein contents in water -soluble and -insoluble fractions of muscle.

(g/100g)

Storage period (h)	refrigeration						Super-chilling					
	3	6	9	12	24	48	3	6	9	12	24	48
Water-soluble fraction	a 2.3±0.1	ad 2.7±0.4	e 3.7±0.1	abf 3.1±0.3	ad 2.6±0.1	ad 2.6±0.2	ad 2.8±0.2	abc 3.0±0.3	be 3.4±0.2	bce 3.4±0.2	d 2.4±0.1	cdf 2.7±0.2
Water-insoluble fraction	b 10.6±1.5	a 8.6±0.5	a 8.6±0.9	a 8.1±0.8	a 8.6±0.2	a 8.7±0.1	a 8.7±0.9	b 10.7±0.2	a 7.2±0.1	a 8.4±0.2	a 8.0±0.2	a 7.5±0.1

Each value is the average±SE. [abcdef]Different letters with each set of data indicate significant difference ($p<0.05$).

for prawns.

Changes in meat extract components The K-value that was calculated from the contents of related nucleic acid components is shown in Fig. 6. The values sharply increased up to 9 h in both storage methods. Then, during refrigeration, they continued to slowly increase. In contrast, the K-value remained constant during super-chilling after storage for 12 h, and a significant difference from the refrigerated samples was observed at 48 h. This result was the result of a slight difference in IMP and Hx contents (data not shown). Since the K-value increases with the reaction of IMPase to such microorganism (Tomioka and Endo, 1984), this activity may be partly inhibited in the super-chilling condition.

Currently, the K-value is considered to be an important standard for judging the freshness of seafood. The K-value of fish immediately after killing is under 10%. Fish meat for raw consumption should be around 20%, and for general sushi use, it should be around 40% (Kato, 1996). Matsumoto and Yamanaka (1990) reported that it would take 4 days at 5℃, 9 days at 0℃, or 12 days at −1℃ for prawns to reach the first stage of decomposition indicated by a putrid smell from the generated putrescine, at 20% of the K-value. Furthermore, Nakamura and

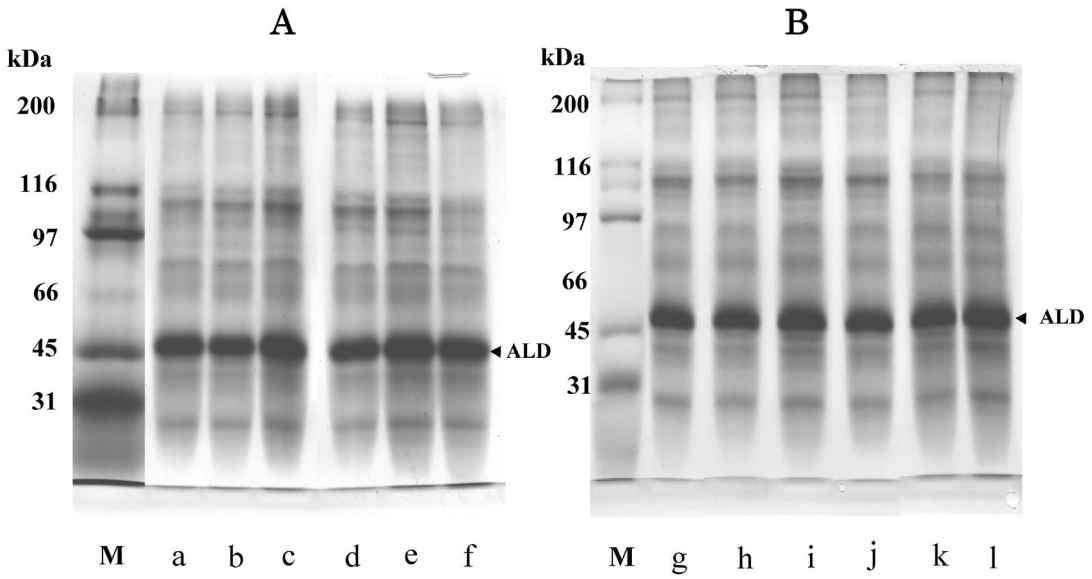

Fig. 8. SDS-PAGE analysis of the water-soluble fractions of meat. (A): refrigeration, (B): super-chilling. M: molecular weight marker, a and g: 3 h, b and h: 6 h, c and i: 9 h, d and j: 12 h, e and k: 24 h, f and l: 48 h. ALD: aldolase.

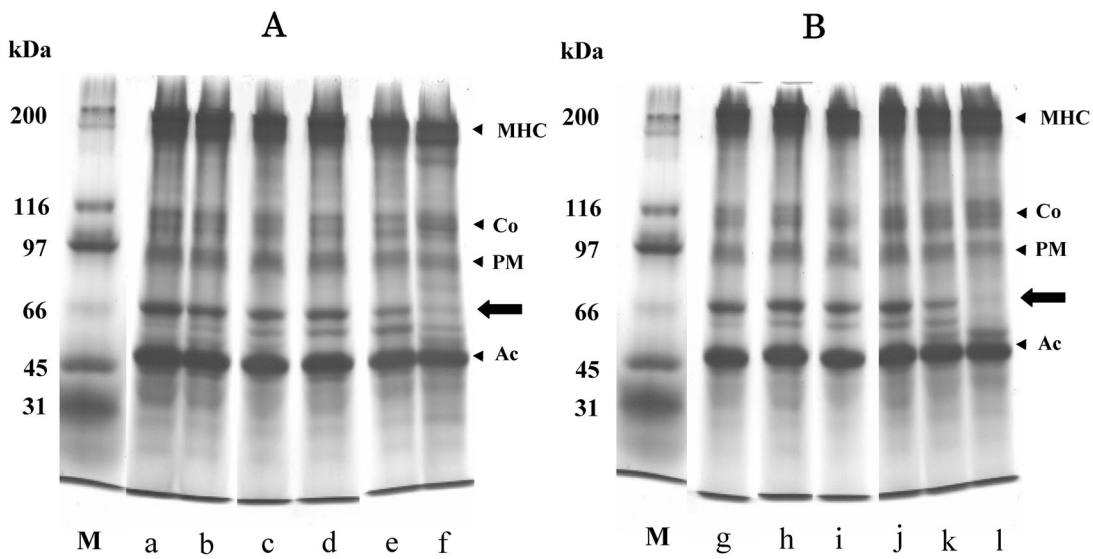

Fig. 9. SDS-PAGE analysis of the water-insoluble fractions of meat. (A): refrigeration, (B): super-chilling. M, molecular weight marker, a and g: 3 h, b and h: 6 h, c and i: 9 h, d and j: 12 h, e and k: 24 h, f and l: 48 h. MHC, myosin heavy chain; Co, collagen, PM, paramyosin, Ac: actin. Arrows indicate unidentified components that fainted during storage.

Ishikawa (1986) reported that it takes 6 days at 2℃ to reach a K-value of 20%. In contrast, the K-value of squid is reported to increase in the early stage of storage (Yokoyama *et al.*, 1994). The value of 40%, which was observed in the present study in squid stored for 48 h, equals the one day stored condition. Such good condition could be maintained longer by super-chilling storage rather than refrigeration.

Although a difference in ATP-related components was observed, free amino acids contents did not show any notable differences between the two storage methods (data not shown). When cells are exposed to low temperatures such as during super-chilling, low molecular weight materials, including amino acids, sometimes increase to resist low temperature stress (Bohnert and Jensen, 1996). However, no difference was observed in the present experiment. This may be because the low temperature stress was relatively small or because the storage time was quite short. In future investigations of detailed temperature settings, changes might be observed in the volume of amino acid in storage at the lowest non-freezing temperature limit.

Although sensory tests were not performed in the present study, there might not be notable differences between the two storage methods because no major difference in meat extracts were observed for IMP, AMP, and free amino acids.

Changes in viable cell counts Figure 7 shows the viable cell counts. In the super-chilling storage, no significant increase in viable cells was observed at 48 h. In contrast, for the refrigerated samples, viable cell counts increased significantly from 24 h to 48 h storage. Therefore, it can be concluded that super-chilling storage effectively depressed the development of microorganisms.

Freezing is an effective storage method for controlling microorganisms. However, drips generated by thawing cause problems, such as the outflow of extractive components (Shirai and Yoshikawa, 1999). Furthermore, freezing is expensive. As super-chilling storage can inhibit the increase of microorganisms without freezing, it is a very effective storage method.

Changes in muscle protein Table 1 shows the change in water-soluble and water-insoluble protein contents. Collagen and connectin, possible proteins for meat softening, are included in the insoluble fraction (Takahashi, 1983). However, no notable changes were observed in the insoluble fraction. Contrastingly, the water-soluble fraction increased during storage from 9 h to 12 h, and decreased after 24 h in both groups. Water-soluble proteins primarily contain carbohydrate catabolic enzymes. The increase of the fraction might be due to the elevation of extraction ability because of the possible weakening of muscle cells. Successful decreasing of the fraction would be due to the coagulation of the proteins because of the change of physiological environment, such as the lowering of pH (Konno and Fukazawa, 1993).

SDS-PAGE analysis also showed no difference or change in band patterns in the water-soluble fractions (Fig. 8). However, a component in the insoluble fraction was distinguished at 48 h in both storage methods (Fig. 9, arrows). The component may become water-soluble, but an identical band is not present in the water-soluble fraction. Therefore, the components may be of a lower molecular weight than 66 kDa. As no changes in physical strength were observed at 48 h storage (Figs. 4 and 5), the lost component would have no relation to meat texture.

Myosin heavy chain and actin did not show any significant change (Fig. 10). In the SDS-PAGE analysis of squid (Kugino *et al.*, 1997) and shrimp (Ando *et al.*, 2004) muscles during chilled storage, no change corresponding to the physical strength was detected. However, using 2% polyacrylamide gel, Kasamatsu *et al.*, (2004) observed that the degraded of connectin during chilled storage corresponding to the change of physical strength.

Collagen bands (Fig. 9, Co) detected in the present study also showed no changes during cool storage. Mizuta *et al.*, (1994) reported that squid muscle has two distinct collagen species, comprising major and minor components. As there were no changes in the major collagen in the present study, there is a possibility that the minor one might have been degraded during storage in the present study. To prove the correlation of collagen and connectin to meat softening, an improved sample preparation method is required.

The results of the present study show that the difference in proteinase action between the two storage temperatures is very small.

Effectiveness of super-chilling storage When squids were refrigerated, unfavorable changes in quality, such as discoloration (Figs. 2 and 3), were observed. In the present study, this problem was overcome by super-chilling storage using a strictly controlled refrigerator. For consumers, the color of fresh foods provides an easily understood index of food freshness, and it may be possible to maintain the color effectively using super-chilling storage. A problem with this technique that remains to be resolved is how to improve the strict temperature control system. At present, the piezo-electric technique can cool capacity of several tens of liters only, and the technology is still at the experimental stage. In addition, effective temperature control for large storage rooms is currently difficult to achieve.

Super-chilling storage is more expensive than refrigeration. However, if the merits shown in this study could be realized at the industrial level, super-chilling storage might be able to provide additional value to commercial foods.

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