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Effect of Super Chilling Storage on Maintenance of Freshness of Kuruma Prawn

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Using natural prawn (*Penaeus japonicus*), a comparison study was conducted between super chilling (−2°C) and cool (5°C) storage for the effectiveness of keeping fish meat fresh. Brightness of the tail color could be retained longer using super chilling storage compared to traditional refrigeration. In addition, a significant color difference in *a* values between the two methods was confirmed when a colorimeter was used. As a physical property of the meat, significant softening was observed after 2 days refrigeration. However, in super chilling storage, no softening was observed even after 3 days of storage. Light microscopy showed the lowering of intercellular connections in the softened meat. The rise in *K*-values was suppressed in super chilling storage. From these results, it can be expected that super chilling storage is able to maintain the color of prawns and the physical properties of the meat, and consequently offers potential as a new storage technique suitable for replacing refrigeration.

Keywords: kuruma prawn, super chilling, refrigeration, freshness, *K*-value, appearance

The quality of foods is heavily dependent on the length of storage. In particular, perishable fish and shellfish can quickly lose the extreme freshness they need to be suitable for human consumption. To help preserve their freshness, such fresh-foods must be stored at low temperatures. Traditionally, seafood has been kept in cold storage or in freezers, but in recent years, an additional storage technique based on the Japanese concept of "Hyo-on" (super chilling) has attracted much interest (Yamane, 1982). Super chilling means a temperature range in which foods remain in a non-frozen condition despite being in sub-zero temperatures. If we try to maintain super chilling temperatures using a regular refrigerator, the necessary temperatures cannot be maintained due to the large temperature fluctuations, and the stored foods freeze partially. Partial freezing generates large ice crystals and cellular structures are destroyed or seriously damaged (Kozima, 1986). To store foods at super chilling temperatures, a specific facility is required to strictly control the temperature, and such a facility is currently under development (Kumagai, 2000).

The freezing point of seafood is considered to be around −2°C and is neither a chilled nor a partially frozen temperature (Yamane, 1982, 1996). When foods are stored at super chilling temperature, the respiratory metabolism of the cells is suppressed and the aging process slows down, resulting in the cells remaining active (Suzuki & Murata, 1999). In addition, when seafood is exposed to temperatures below 0°C, it suffers from low temperature stress, and it is believed that the free amino acid and sugar content of the cells increase in order to resist being frozen (Bohnert & Jensen, 1996). Furthermore, being kept at lower temperatures leads to suppression of the proliferation of harmful microorganisms. In other words, storing food at super chilling temperature has three distinct advantages: maintaining food freshness, retaining high food quality, and suppressing harmful microorganisms. Innovative super chilling techniques have also been developed: super chilling-drying, -aging, -fermentation, and -condensation, and these techniques have been applied for processing of fresh foods (Yamane, 1996). In addition to food applications, the technique has been applied to the preservation of organs for transplantation, and further developments in these research areas are expected in the near future (Shiba *et al*., 1998).

At present, the rapid deterioration of seafood remains a serious problem with regard to seafood distribution and safety. With the increase of imported fresh food, it is very important to provide consumers with high quality products by prolonging the period of freshness. As for super chilling storage on seafood. There have yet been few investigations into super chilling storage on seafood, and there are many problems yet to be resolved (Fukuma *et al*., 1998; Ayaki *et al*., 1999; Ando *et al*., 2002).

Prawns are one of the most important shellfish imported to Japan. However, their freshness rapidly deteriorates with the their high protease activity (Lindner *et al*., 1988). In addition, their body color turns blackish and presents another challenging problem (Koburger *et al*., 1985). The purpose of our study was to determine the effect of super chilling storage on maintaining the freshness of prawn in comparison with regular refrigeration.

Materials and Methods

Storage conditions and preparation of samples Six live prawns were purchased from a city market and were stored for 3 days at −2°C (super chilling storage) and 5°C (cool storage), respectively. An incubator (NH-60S, Ninomiya Sangyo Co., Tokyo) was used for the super chilling storage. The incubator does not incorporate a cooling fan to keep an even temperature in the box and uses a piezo-electric element rather than a compressor for cooling, which avoids the effect of vibration interference. Temperature fluctuation of the incubator is less than 0.1°C. In the present study, kuruma prawn meat did not freeze even though the super chilling temperature (−2°C) was near its freezing point

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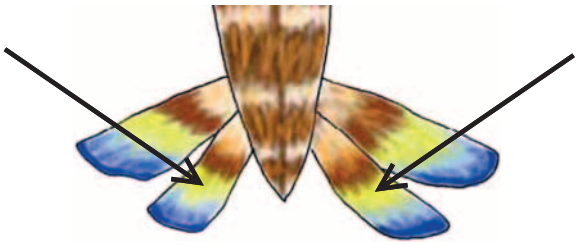

Fig. 1. The area where color was measured. Arrows indicate the parts where color was measured a colorimeter.

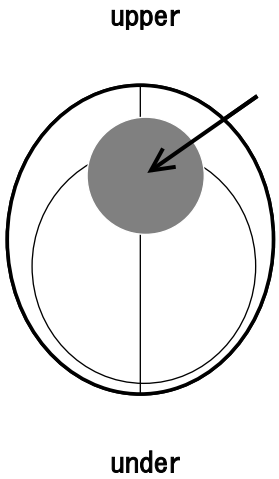

Fig. 2. Illustration of transverse section of muscle. Arrow indicates the area where firmness was measured.

(−2.2°C) (Ruello, 1977).

Evaluation of external appearance The change in tail color was evaluated by a colorimeter (Z-300A, Nihon Densyoku Kogyou Co., Tokyo). The measuring points are shown in Fig. 1 (arrows).

Measurement of physical strength The physical strength of meat was measured by a rheometer (RE2-33005S, Yamaden Co., Tokyo) equipped with a cylindrical plunger (5 mm in diameter). The prawn heads and shells were removed at selected time intervals, and the meat was sliced to a thickness of 10 mm. The plunger pierced the meat parallel to the body axis at a stage speed of 1 mm/s. The measured area is shown in Fig. 2 (arrow). The strength at 2 mm deformation was measured as the physical strength of the meat.

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

K-value Meat extract was obtained by perchloric acid preparation, and ATP related components in the extract were determined by HPLC (Ando *et al*., 1998). *K*-value was measured from the contents of ATP related components.

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

Determination of water-soluble and water-insoluble proteins Three grams of meat was homogenized (15,000 rpm, 1 min) with 10 volumes of cold water and centrifuged (10,000 rpm, 15 min). Protein concentration in the supernatant (water-soluble protein) was determined by the method of Lowry *et al*. (1951) and that in the residue (water-insoluble protein) was determined by micro-Kjeldahl method.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) Analysis of the protein components was carried out using the method of Laemmli (1970). 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 min. The water-insoluble fraction (about 30 mg) was put in SDS-sample buffer (300 μl) and heated in boiling water for 5 min. For both samples, a portion containing 4 μg protein was loaded in one lane of the stacking gel. Bovine IgG was used as a standard protein. 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.

Statistical analysis Statistical differences of the results were determined by analysis of variance using the calculation software, Excel 2000 (Microsoft Co., USA).

Results and Discussion

Change of tail color Change of tail color was evaluated by a colorimeter (Fig. 3). No significant differences in *L* and *b* values were observed between the two storage temperatures, nor did *a* value change for the super chilling storage samples, while a significant rise was observed after 2 days storage for cool stored samples. Takehara and Nakahara (1970) reported that *a* value was positively correlated to the increase of a chestnut brown color of red-tea. In other words, the significant rise of *a* value in the cool stored samples (Fig. 3) was affected by the light brownish color.

Raw prawn and crab body color turns brownish or blackish during cool storage and their market value falls significantly. This color change is caused by melanin generated by tyrosinase action (Koburger *et al*., 1985; Savagaon & Sreenivasan, 1986). Sodium bisulfate is sometimes used to suppress these color changes; however, considering the residual sulfur dioxide obtained using these methods (Mcevily *et al*., 1991), food additives should be kept to a minimum for human health. Our experimental results show that the above-mentioned enzymatic reactions can be inhibited by simply controlling storage temperature. It is therefore proven that super chilling storage is a very effective technique for storing fresh food without using preservatives.

Changes in meat texture Figure 4 shows the changes in meat texture. The breaking force was decreased from 2.4 N to 1.0 N in the refrigerated samples over 2 days. In contrast, after 3 days super chilling storage, the firmness values remained unchanged. There was thus a significant difference between the two samples at 2 and 3 days storage. These results indicate the suppression of meat softening during storage by super chilling storage.

The softening phenomenon observed in cool stored samples could be caused by the various enzymes that exist in the meat.

Generally, seafood meat is considered to become softer with the reduction of freshness, and the cause of this phenomenon is reported to be due to the disintegration of collagen or connectin by endogenous proteinase in cool storage (Kugino *et al*., 1997; Ando *et al*., 1999; Kagawa *et al*., 2002). Since softening of the seafood meat was suppressed by using super chilling storage in the present study, disintegration of collagen and connectin might therefore be suppressed at the super chilling temperatures.

Histological observations Figure 5 shows the results obtained by light microscopy. No significant differences were observed between the two temperatures during the first day of storage (Figs. 5A, B and C). However, on the second day, intercellular spaces appeared in the refrigerated samples (Fig. 5D). On the third day, similar spaces were also observed (Fig. 5F). In contrast, super chilling samples showed no histological changes in their cells and no intercellular spaces were observed by the third day (Fig. 5G). These results closely correlated with the changes in physical properties (Fig. 4). Mizuta *et al*. (1997) reported similar findings. In squid meat also, intercellular spaces appear when the meat becomes softer during cold storage (Ando *et al*., 1999). The reason for this phenomenon is thought to be the result of the intercellular connectivity becoming lower during cold storage. The osmotic pressure used for the fixing liquid in this experiment (550 mOsm (5% glutaraldehyde) + 200 mOsm (0.1 M phosphate buffer) = 750 mOsm) (Maser *et al*., 1967) was higher than that of the mammalian cell (300 mOsm) and thus the muscle cells might have contracted (Maser *et al*., 1967). At an earlier stage, integrity among muscle cells can resist the contracting force. However, with the lowering of binding force, muscle cells would separate from each other. These results show that super chilling storage inhibits the deterioration of intercellular connectivity. A major element of muscle cellular connection is collagen (Takahashi, 1983), and Mizuta *et al*. (1997) showed that a minor component of muscle collagen disintegrates with increasing length of cold storage correlating to meat softening. It is assumed that storage at super chilling temperature could inhibit such a disintegration of collagen, and result in suppressing the meat softening.

Free amino acid contents Table 1 shows the free amino acid contents. Pro, Gly, Ala, and Arg, which relate to the taste of prawns (Ohta, 1991), and Tau, Glu, and $GluNH_2$ were contained in the greatest quantities. Among them, the amount of Pro, Gly, Ala, Tau, and $GluNH_2$ showed no significant changes for either temperature. Glu decreased with increase of storage time for both temperatures while Arg showed a tendency to increase for both, Glu acts as an amino group donor in many reactions, being a substrate for aminotransferase in the living body (Dinwoodie & Boeker, 1979), and thus Glu may have decreased by this reaction. In contrast, Arg is involved in both the urea and TCA cycles, but the cause of the increase in this experiment was not clear. In any case, the contents of free amino acids showed no significant changes during super chilling storage. When cells are exposed to low temperatures, as in this experiment, there is a tendency for the lower molecular materials such as amino acids in the cells to increase in order to resist low temperature stress (Bohnert & Jensen, 1996). However, such a phenomenon was not observed this time. The reason could be that either the low temperature stress was too little or the storage period was too short. The amount of amino acids might change in future experiments by using the lowest temperature at which the samples are not frozen.

According to the report made by Matsumoto and Yamanaka (1990a), most of the free amino acids contained in prawns were Tau, Glu, Pro, Gly, Ala and Arg. They decrease during the early storage period except for Tau, but increase in the prolonged period, at the first decomposition (Matsumoto & Yamanaka, 1990a). In our experiments, since decomposition was not observed, it was thought the samples were not at the stage of decomposition. In addition, when lobster was stored at 5°C, Pro, Gly, Ala and Arg contents showed no change (Shimada *et al*., 1998). This

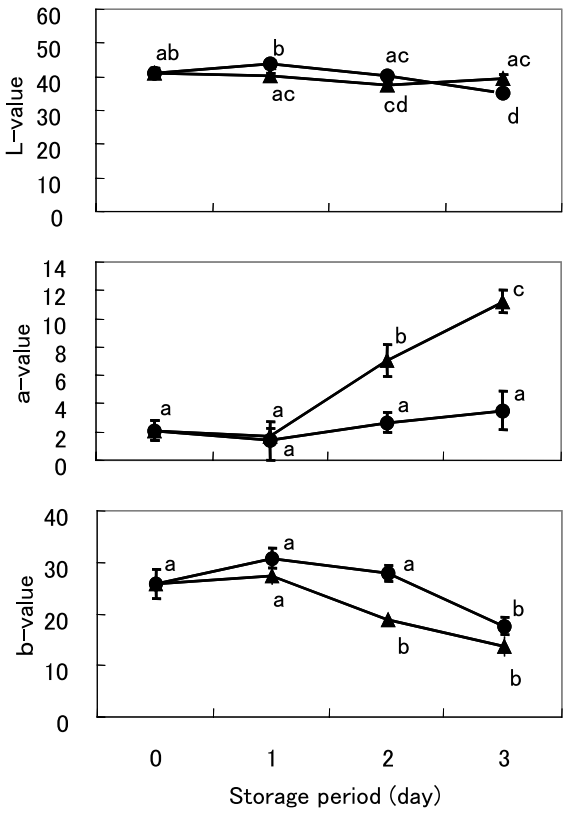

Fig. 3. Change in tail color during storage. Each value is the average of six determinations. [abcd]Different letters with each set of data indicate significant difference ($p<0.05$). ▲ 5°C storage, ● −2°C storage.

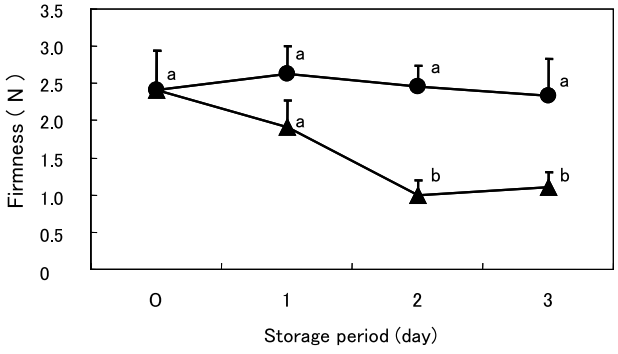

Fig. 4. Change of muscle firmness during storage. Each value is the average of six determinations. [ab]Different letters with each set of data indicate significant difference ($p<0.05$). ▲ 5°C storage, ● −2°C storage.

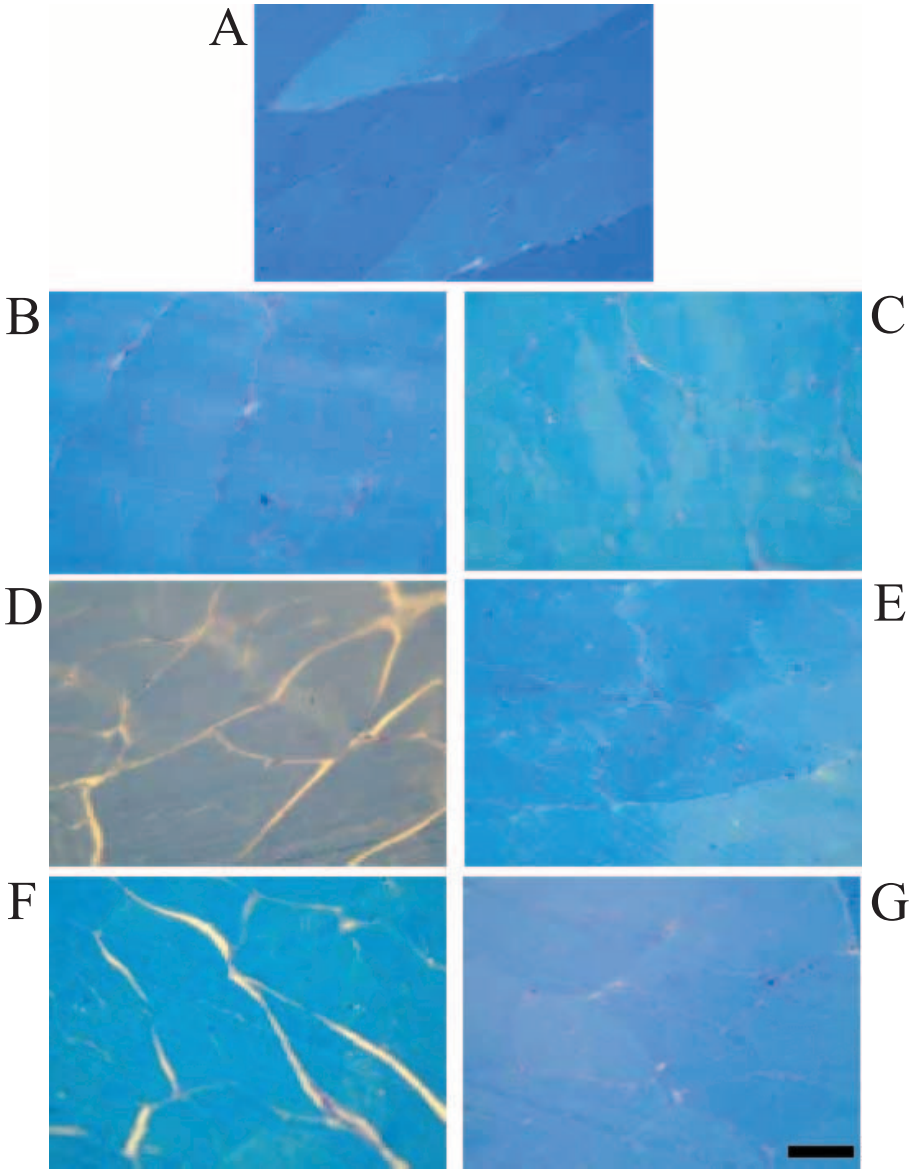

Fig. 5. Optical microscopic photographs of muscle. A, just after death; B, 1 day storage at 5°C; C, 1 day storage at −2°C; D, 2 days storage at 5°C; E, 2 days storage at −2°C; F, 3 days storage at 5°C; G, 1 day storage at −2°C. Bar represents 50 μm.

result suggests that the species of prawn would also influence the contents of free amino acids in addition to storage temperature.

According to the results of textural measurement and histological observation, enzymatic activity would be different between the two temperatures. However, the minimal difference in free amino acid content shows that peptidase activities which generate free amino acids might not differ between the two temperatures.

Change in K-value Figure 6 shows the results of the change in *K*-value. No difference was observed for either sample treatment after 1 day's storage. However, after 2 days, the value of the cool stored samples rose to 7.3% and for the super chilling stored ones to 2.2% on the third day, the cool stored samples rose to 15.5% and the super chilling stored ones to 7.9%. These results indicate a significant difference between the two methods. Since the *K*-value rises dependent on IMPase reactions in microorganisms (Tomioka & Endo, 1984), it is thought that the reactions were controlled in the super chilling condition.

Currently, the *K*-value is believed 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 2% and for general sushi use, it should be around 4% (Kato, 1996). Matsumoto and Yamanaka (1990b) reported that it would take either 4 days at 5°C, 9 days at 0°C or 12 days at −1°C for prawns to reach the first stage of decomposition as shown by a putrid smell from the generated putrescine, at 20% of the *K*-value. Also, Nakamura and Ishikawa (1986) reported that it takes 6 days at 2°C to reach the *K*-value of 20%. Based on these reports, it may be that the samples of the present study had not reached the decomposition state during the storage period. However, the cool stored samples smelled unpleasant after only 2 days storage (data not shown). This smell may have been due to volatile materials such as trimethylamine. In contrast, the super chilling storage samples smelled less. On the basis of these

Table 1. Contents of free amino acids in the muscle during storage. (μmol/g)

		5°C storage			−2°C Storage		
Storage period (day)	0	1	2	3	1	2	3
P-ser	0.228	0.162	0.158	0.266	0.223	0.145	0.206
Tau	16.490	16.739	19.153	19.262	19.687	17.468	19.331
UREA	7.147	6.541	7.406	7.474	6.805	6.141	8.537
Asp	0.242	0.227	0.151	0.157	0.251	0.161	0.160
Thr	1.269	1.753	1.579	1.285	1.308	1.137	1.374
Ser	2.991	3.612	3.896	4.255	3.846	2.754	3.999
$AspNH_2$	1.828	2.498	2.036	1.933	3.628	1.858	1.967
Glu	16.223	7.301	2.657	3.143	8.815	3.219	2.435
$GluNH_2$	10.396	11.392	10.622	9.681	11.325	9.370	10.104
Sar	0.300	0.383	0.642	0.586	0.513	0.000	0.566
α-AAA	0.000	0.194	0.327	0.000	0.131	0.221	0.301
Pro	25.528	37.070	27.110	31.588	32.757	29.155	30.099
Gly	111.549	81.797	95.919	78.740	73.221	74.500	83.993
Ala	7.172	8.177	8.028	8.703	6.642	8.047	7.767
Cit	0.121	0.232	0.133	0.186	0.129	0.148	0.186
α-ABA	0.127	0.168	0.210	0.177	0.107	0.165	0.161
Val	1.502	1.970	1.943	1.718	1.431	1.821	1.601
Cys	0.127	0.202	0.149	0.101	0.100	0.127	0.163
Met	0.734	0.990	1.005	0.929	0.767	0.975	1.051
CysSH	0.191	0.108	0.181	0.115	0.119	0.110	0.108
Ile	0.734	0.924	1.000	0.838	0.755	0.855	0.832
Leu	1.187	1.783	1.616	1.483	1.380	1.449	1.474
Tyr	0.735	1.005	0.964	0.673	0.721	0.943	0.729
Phe	0.451	0.668	0.653	0.517	0.401	0.486	0.439
β-Ala	0.206	0.091	0.142	0.139	0.161	0.117	0.146
β-AiBa	0.000	0.000	0.000	0.000	0.075	0.000	0.000
γ-ABA	0.000	0.000	0.087	0.000	0.000	0.000	0.113
Trp	0.000	0.000	0.000	0.159	0.000	0.000	0.000
$EOHNH_2$	0.378	0.369	0.584	0.795	0.664	0.586	0.679
NH_3	2.378	2.545	4.477	7.397	4.297	3.306	4.280
Orn	0.212	0.247	0.380	0.671	0.433	0.340	0.554
Lys	1.251	2.304	1.423	1.286	1.728	1.521	1.504
His	0.910	1.047	1.018	0.849	0.778	1.081	0.711
3Mehis	0.154	0.195	0.182	0.124	0.191	0.170	0.114
Arg	27.482	51.078	62.811	52.201	45.626	52.222	60.694

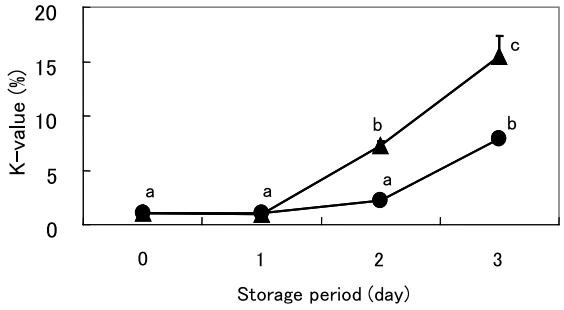

Fig. 6. Change in *K*-value of muscle during storage. Each value is the average of six determinations. [abc]Different letters with each set of data indicate significant difference ($p<0.05$). ▲ 5°C storage, ● −2°C storage.

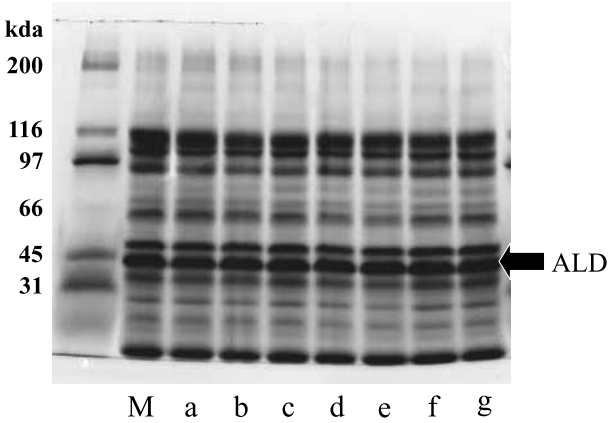

Fig. 7. SDS-PAGE analysis of the water-soluble fraction of muscle. a, just after death; b, 1 day storage at 5°C; c, 1 day storage at −2°C; d, 2 days storage at 5°C; e, 2 days storage at −2°C; f, 3 days storage at 5°C; g, 3 days storage at −2°C; M, molecular weight marker. ALD, aldolase.

results, super chilling storage may potentially be a more effective storage technique than traditional refrigeration.

Changes in muscle protein Table 2 shows the changes in water-soluble and insoluble protein contents. Collagen, and connectin, possible proteins for meat softening, are included in the insoluble fraction (Takahashi, 1983). Both increases and decreases were observed, however, there was no specific tendency for either treatment group.

Electrophoresis also showed no difference or change in band pattern for either group (Figs. 7 and 8). Therefore, it can be assumed that no significant protein decomposition occurred for either treatment temperature.

If collagen and connectin suffered any degradation, it should have been detected in electrophoresis of non-soluble fractions (Fig. 8). However, connectin has a molecular weight of 2000 kDa, which is too large to detect in this electrophoresis. Collagen bands (Fig. 8, Co) detected in this study are major components of muscle collagen and do not change during cool storage (Mizuta

Table 2. Protein contents in water-soluble and water-insoluble fractions of muscle. (g/100 g)

		5°C storage			−2°C storage		
Storage period (day)	0	1	2	3	1	2	3
Water-soluble fraction	5.2 ± 0.1^{a}	4.4 ± 0.2^{b}	5.5 ± 0.1^{ac}	5.9 ± 0.1^{c}	4.5 ± 0.2^{b}	5.3 ± 0.2^{a}	5.0 ± 0.2^{a}
Water-insoluble fraction	10.9 ± 0.4^{ac}	11.0 ± 0.3^{ac}	12.3 ± 0.3^{b}	10.4 ± 0.5^{c}	11.8 ± 0.2^{ab}	11.1 ± 0.2^{abc}	11.9 ± 0.4^{ab}

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

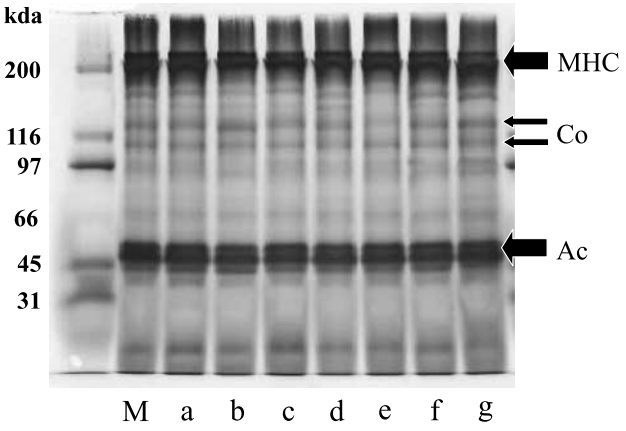

Fig. 8. SDS-PAGE analysis of insoluble fraction of muscle. a, just after death; b, 1 day storage at 5°C; c, 1 day storage at −2°C; d, 2 days storage at 5°C; e, 2 days storage at −2°C; f, 3 days storage at 5°C; g, 3 days storage at −2°C; M, molecular weight marker. MHC, myosin heavy chain; Ac, actin; Co, collagen.

et al., 1997). Additionally, the content of a minor component of collagen, reported to disintegrate during storage (Mizuta *et al*., 1997), is too small to detect by the present method. To confirm there is a correlation of collagen and connectin to meat softening, the sample preparation method would have to be improved.

Although myofibrillar proteins, which account for 60 to 70% of prawn meat protein, denature easily, sarcoplasmic proteins, which account for 20 to 30% of prawn meat protein, are relatively stable (Koyama, 1987). Shaban *et al*. (1987) reported that the sarcoplasmic proteins remained unchanged but the myofibrils decreased and the alkali-soluble proteins increased when prawns were stored at −20°C for 1 to 3 months. These changes were detected as new bands in SDS-PAGE. However, in our experiment, water-soluble proteins did not show any changes (Table 2, Fig. 7). Therefore, changes in muscle protein would be very faint during the storage periods used in this experiment.

These results show that the difference in proteinase activities between −2°C and 5°C stored muscles would be very small.

Effectiveness of super chilling storage When prawns were refrigerated, unfavorable changes in quality such as discoloration (Fig. 3), deterioration of texture and a rapid rise in *K*-value (Fig. 6) were found. In our study, these problems could be overcome by super chilling storage using the strictly controlled refrigerator. For consumers, the color or texture of fresh foods provides an easily understandable index for food freshness, and it may be possible to maintain these effectively by using a super chilling temperature. A problem of 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 level. In addition, effective temperature control for a large storage room is currently difficult to achieve. However, in the near future when these problems are resolved, super chilling storage is expected to offer an effective alternative storage technique to refrigeration.

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