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Author(s)	UMEDA, Norio; KOSUGI, Tetsuya; KITAZAWA, Hiroyuki et al.
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Changes in ATPase Activity and Solubility of Carp Myofibrils during Frozen Storage under Three Different Buffer Conditions

Norio UMEDA[1], Tetsuya KOSUGI[1], Hiroyuki KITAZAWA[1], Norio INOUE[2] and Haruo SHINANO[1]

Abstract

The effects of storage temperature (at −4, −11, and −26°C) and buffer concentration (5 and 50 mM) on the solubility and ATPase activity of carp myofibrils were compared among three different kinds of buffer. Three kinds of buffer solution, Tris-HCl, Tris-maleate, and phosphate, in the absence of additional neutral salts, were used for this experimental system. Changes in the solubility and ATPase activity differed among the three different buffers in the 50 mM concentration. The extent of decrease in solubility and ATPase activity in the 50 mM system was smallest with the Tris-maleate and phosphate buffers, respectively. When the myofibrils were stored with Tris-HCl buffer, both the indices for denaturation markedly decreased within 3 weeks. The decrease in solubility prior to ATPase inactivation in the phosphate buffer was similar to the freeze denaturation pattern reported in fish muscle. In the case of 50 mM Tris-maleate buffer, ATPase activity decreased while still retaining solubility. Observations of storage temperature dependence in both the indices suggest that the storage temperature affects on the mobility of water and the volume of unfrozen solution, and the former relates intensively to the freeze denaturation of myofibrils.

Key Words: Carp, Myofibrillar protein, Solubility, ATPase activity, Frozen storage, Freeze denaturation

Introduction

Changes in the pH of fish muscle have been considered to be one of the causative factors in the denaturation of fish protein during frozen storage (Fennema 1973a). However, it was reported that the extent of changes in pH in frozen fish flesh was small, as the buffer action of unfrozen liquid was quite strong, and the pH change would therefore not be the main factor in protein freeze denaturation (Ohta and Tanaka, 1978).

Recently, Kitazawa et al. (1995) reported that the decrease in solubility was larger at higher storage temperatures in an experimental system of phosphate buffer (K_2HPO_4-NaH_2PO_4, pH 6.8) and in the absence of neutral salt. This temperature dependence was quite similar to that observed in fish flesh (Connell, 1960). It could be considered that this phosphate buffer was concentrated and unfrozen at a storage temperature of −4°C, because both K_2HPO_4 and NaH_2PO_4 which comprised the

[1] Laboratory of Food Wholesomeness, Faculty of Fisheries, Hokkaido University (北海道大学水産学部食品機能化学講座)
[2] Laboratory of Biochemical Process Technology, Faculty of Fisheries, Hokkaido University (北海道大学水産学部生物化学工学講座)

buffer were not at saturation point [their eutectic points are −13.7 and −9.7°C, respectively (Fennema, 1973b)]. It is suggested that the extent of changes in pH value of the unfrozen solution may be small in the above experiment. This supports the idea that the decrease in solubility is caused by other factors except the pH changes of the model system.

The Tris-maleate buffer (near neutral pH range) has been used for the experimental model system of freeze denaturation. However, the eutectic points of the constituents and the pH changes of the system have never been examined and discussed. Therefore, we compare the ATPase activities and solubilities in the freeze denaturation patterns of carp myofibrils among the three different buffer systems, such as Tris-mareate, Tris-HCl and phosphate. The eutectic points of the salts and buffers used in these experiments were simultaneously measured, and the changes in pH under frozen conditions were discussed.

Materials and Methods

Fish

Carp *Cyprinus carpio* (average weight, 1 kg) were used for this experiment.

Preparation of myofibrils

Myofibrils were prepared from dorsal lateral muscles of live carp using the same method of Kitazawa et al. (1995). Buffer solutions used for preparation were 5 and 50 mM Tris-HCl, Tris-maleate, and phosphate buffers (pH 6.8).

Conditions of freezing, storage, and thawing

Each 8 ml portion of the above myofibril suspension was placed in a polypropylene tube (18×150 mm). The tubes were frozen to the prescribed temperatures (−4, −11, and −26°C) in a bath of ethanol solution mixed with solid carbon dioxide, and then placed in freezers adjusted to each temperature. Samples were removed from the freezer at intervals on the appropriate days and thawed in an incubator at 25°C. KCl was added to each sample to adjust it to 0.6 M in the final concentration, and dissolved. The samples were kept in an ice bath for 2 h with constant stirring to extract salt soluble proteins and then dialyzed against 0.6 M KCl-20 mM Tris-HCl buffer (pH 7.0) for 24 h (4°C). The inner solution of the dialysis was homogenized with a Teflon pestle homogenizer and submitted to the following analysis. The samples before freezing, as 0 day samples, underwent the same procedure.

Protein and solubility determination

Protein concentration was determined by the biuret method (Gornall et al. 1949) using bovine serum albumin as a standard.

Solubility (%) was the ratio of proteins in the supernatant after centrifugation (20,000×g, 15 min) to those before centrifugation.

ATPase activity

Ca^{2+} (60 mM KCl)- and EDTA-ATPase activity were both assayed using the method of Takatori et al. (1992), except that a 20 mM Tris-HCl buffer (pH 7.0) was

used.

Measurements of eutectic point and pH

The eutectic points were determined by measuring the thawing points of the salt and buffer solutions with a differential scanning calorimeter (DSC) (Rigaku Denki Co., PTC-10A). The measurement temperature started at −40°C, increasing to 5°C, at 2.0 °C/min increments.

The pH value of the thawed myofibril suspension was measured by a pH meter (Horiba-H7 SD).

Results

pH

Under all conditions examined throughout frozen storage, hardly any changes in the pH of the myofibril suspensions were observed after thawing (Fig. 1).

Solubility

The solubility of the myofibrils stored at −4 and −11°C with 50 mM Tris-HCl buffer decreased to about 25% after 3 weeks, with very little change occurring during subsequent storage (Fig. 2A). The extent of the decrease in solubility at −4°C was greater than that at −11°C. The solubility particularly decreased to about 30% after a week in 5 mM buffer at −4 and −11°C. In the case of 50 mM at

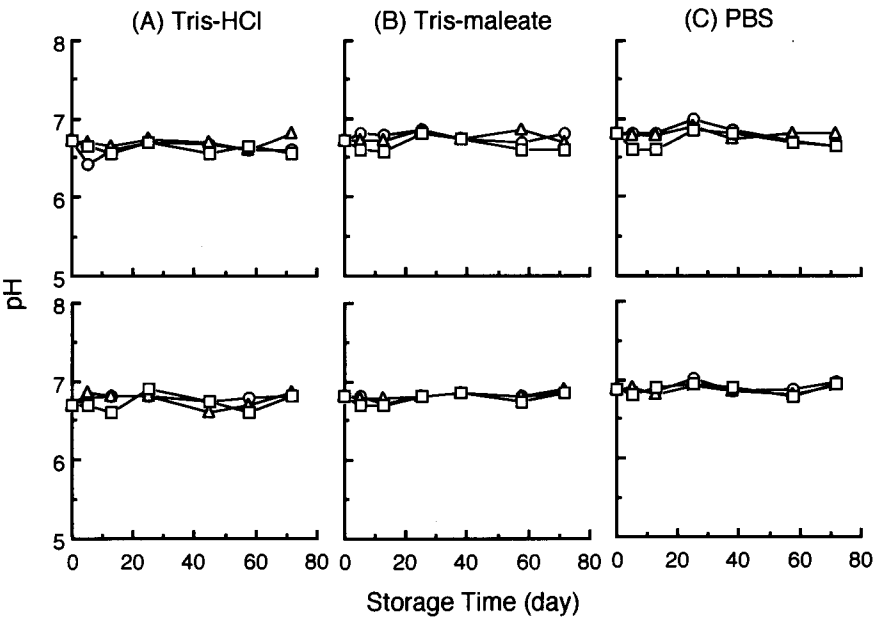

Fig. 1. Changes in pH of carp myofibrils during frozen storage.
The myofibrils were suspended in 5 mM (upper frames) and 50 mM (lower frames) buffers (pH 6.8), and stored at −4°C (○), −11°C (△), and −26°C (□).
(A), Tris-HCl buffer; (B), Tris-maleate buffer; and (C), phosphate buffer (PBS).

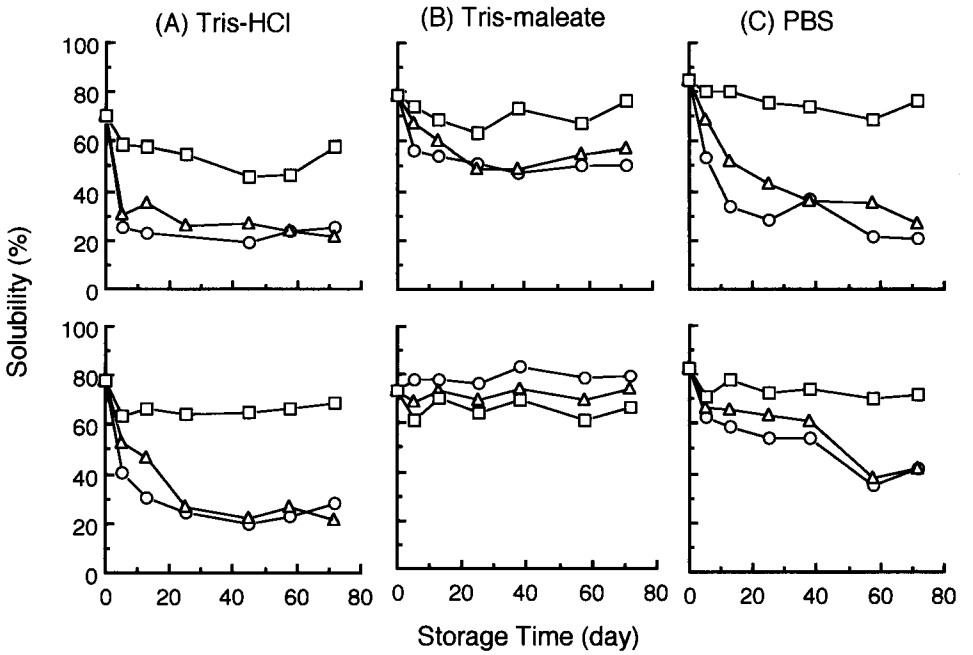

Fig. 2. Changes in solubility of carp myofibrils during frozen storage.
The myofibrils were suspended in 5 mM (upper frames) and 50 mM (lower frames) buffers (pH 6.8), and stored at −4°C (○), −11°C (△), and −26°C (□).
(A), (B), and (C) are the same as in Fig. 1.

−26°C, the solubility remained at about 65% from the 5th to the 72nd day (final day) of storage. The solubility in 5 mM buffer at −26°C was 47-65% over the same storage period.

As for the solubility of myofibrils stored with Tris-maleate buffer (Fig. 2B), that of 5 mM buffer at −4 and −11°C decreased to about 50% after 3 weeks, and remained at this level during the subsequent storage period. In the case of 5 mM buffer at −26°C, the solubility was 65-75% from the 5th to the 72nd day of storage. No significant decrease in solubility was observed in 50 mM buffer at −4°C during frozen storage, and the levels of solubility at −11 and −26°C remained about 70 and 60-70% during the 5th to the 72nd day of storage, respectively. The extent of the decrease in solubility at −4 and −11°C was smaller than those of the other two buffers examined.

In the case of the phosphate buffer (Fig. 2C) at −4 and −11°C, the extent of the changes in solubility in the 5 mM concentration was larger than those observed in the 50 mM concentration throughout the 38 days of storage. Under 50 mM buffer conditions, a noticeable decrease was observed by the 58th day.

ATPase activity

The changes in Ca^{2+} (60 mM KCl)-ATPase (Fig. 3) and EDTA-ATPase (Fig. 4) activities were both quite similar. Henceforth, both the changes are described as the changes in ATPase activity in this paper.

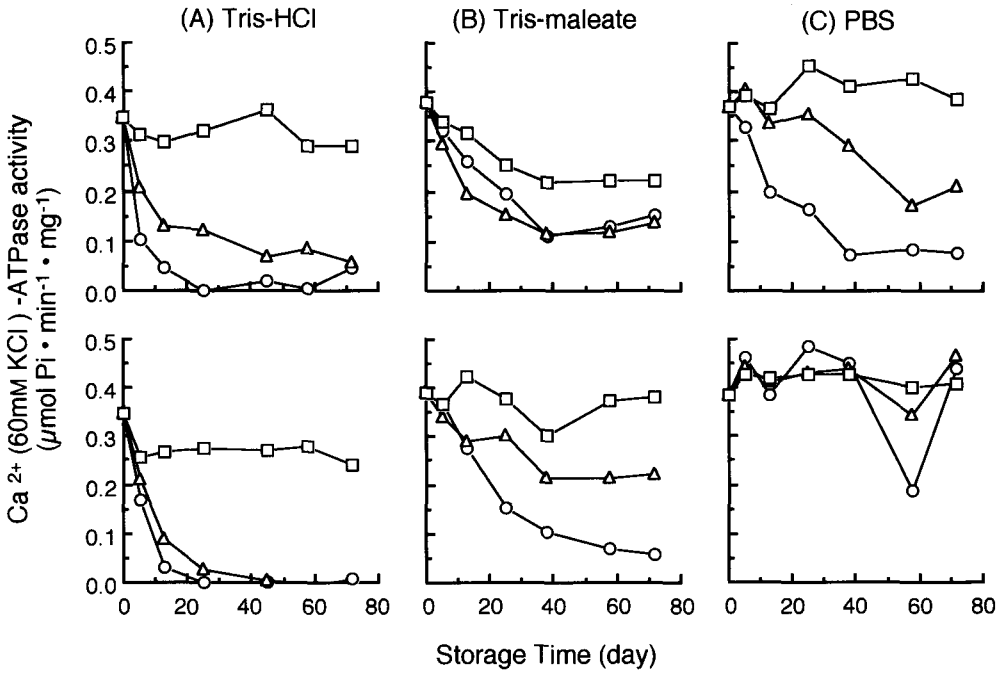

Fig. 3. Changes in Ca^{2+} (60 mM KCl)-ATPase activity of carp myofibrils during frozen storage. The myofibrils were suspended in 5 mM (upper frames) and 50 mM (lower frames) buffers (pH 6.8), and stored at −4°C (○), −11°C (△), and −26°C (□). (A), (B), and (C) are the same as in Fig. 1.

In the case of the Tris-HCl buffer, the extent of the decrease in ATPase activity for myofibrils stored at −26°C (Figs. 3A and 4A) was smallest among the temperatures examined, and the extent of the decrease in activity at −4°C was larger than that at −11°C (in both the 5 and 50 mM concentrations). The temperature dependence of ATPase inactivation agreed with that of solubility.

The extent of the decrease in ATPase inactivation with 50 mM Tris-maleate buffer was larger with an increase in storage temperature (Figs. 3B and 4B). In the case of 5 mM Tris-maleate buffer, ATPase activity lost about 42% of its original value within 38 days of storage at −26°C.

In the case of the 50 mM phosphate buffer (Figs. 3C and 4C), no difference in myofibrillar ATPase activity among the three storage temperatures was observed over the storage period. ATPase activity decreased suddenly on the 58th day at −4 and −11°C; however, no clear explanation can be given regarding this irregular decrease. In the case of the 5 mM concentration, the extent of inactivation was larger at higher storage temperatures.

Discussion

Ohnishi et al. (1978) reported that the rate of decrease in solubility and the extent of structural changes in actomyosin observed using an electron microscope

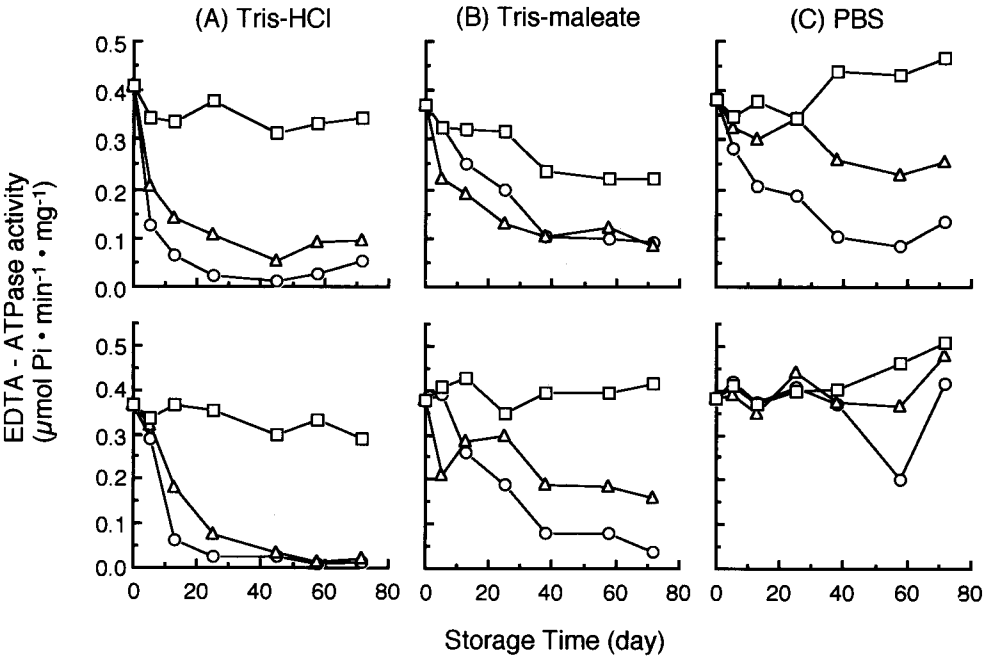

Fig. 4. Changes in EDTA-ATPase activity of carp myofibrils during frozen storage.
The myofibrils were suspended in 5 mM (upper frames) and 50 mM (lower frames) buffers (pH 6.8), and stored at −4°C (○), −11°C (△), and −26°C (□).
(A), (B), and (C) are the same as in Fig. 1.

were greatest in acidic pHs, when the pHs of experimental solutions ranged between 5.8 and 9.4 before freezing. In this case, buffers were not used to avoid complications in analysis caused by the presence of a buffer. In general, when a buffer is used in an experiment, it should be considered that the dissociation constants and activity coefficients of salts used depend on the temperature of the solution (Perrin and Dempsey, 1974) and that the pH changes when the temperature of the unfrozen solution reaches its specific eutectic point (Fennema, 1973b). Actually, the effects of these factors on freeze denaturation have not yet been examined, due to the difficulties in confirming those during frozen storage. In this study, the eutectic point was taken into consideration and a buffer which undergoes only small pH change under frozen conditions was selected, as it had been reported that the effect of pH on the freeze denaturation of fish muscle was negligible during frozen storage (Ohta and Tanaka, 1978). The eutectic points of Tris and maleic acid were −6.2 and −6.9°C, respectively, when measured by a DSC (Fig. 5). The eutectic points of hydrochloric acid, K_2HPO_4, and NaH_2PO_4 are −86 (Perry, 1934), −13.7, and −9.7°C (Fennema, 1973a), respectively. Consequently, when considering the eutectic points of these acids and salts, it is surmised that the three different buffers used in this experiment were concentrated and presented in an unfrozen state under −4°C storage conditions.

The Tris saturation point of Tris-HCl buffer and the eutectic point of Tris-

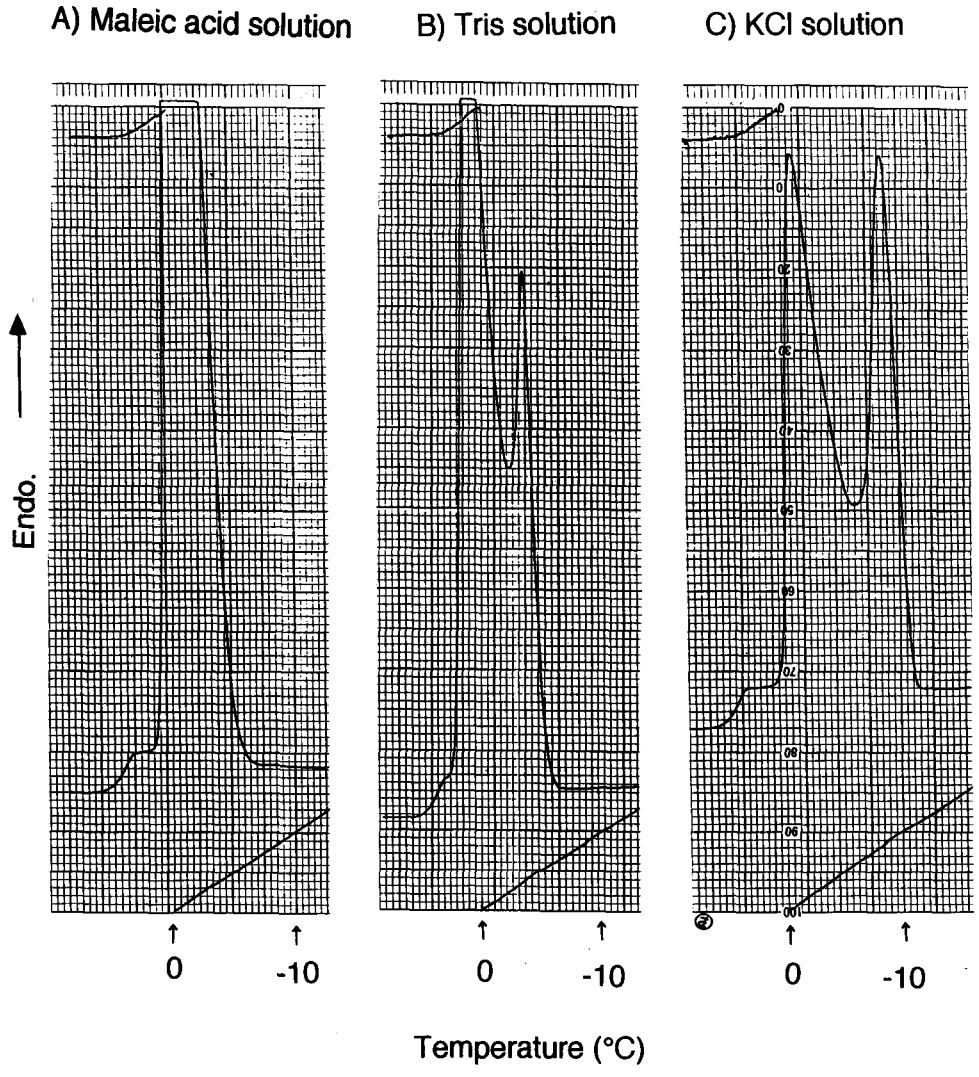

Fig. 5. Differential scanning calorimetry thermograms of maleic acid (A), Tris (B), and KCl (C) solutions.

maleate buffer were −17.0 and −13.0°C, respectively, when measured by a DSC (Fig. 6). The eutectic point of phosphate buffer was estimated to be about −15.4°C by extrapolating the thawing point of the solution when the mole fraction of KCl in phosphate buffer was zero (Fig. 7). Therefore, it was assumed that the buffers were concentrated and might be in an unfrozen state at −11°C. With a decrease in temperature, it appears that the changes in the dissociation constants and activity coefficients, and with an accompanying pH at −11°C were greater than those at −4°C. The extent of changes in the indices at −11°C, however, was the same or smaller than that at −4°C. The decrease in solubility and the inactivation of

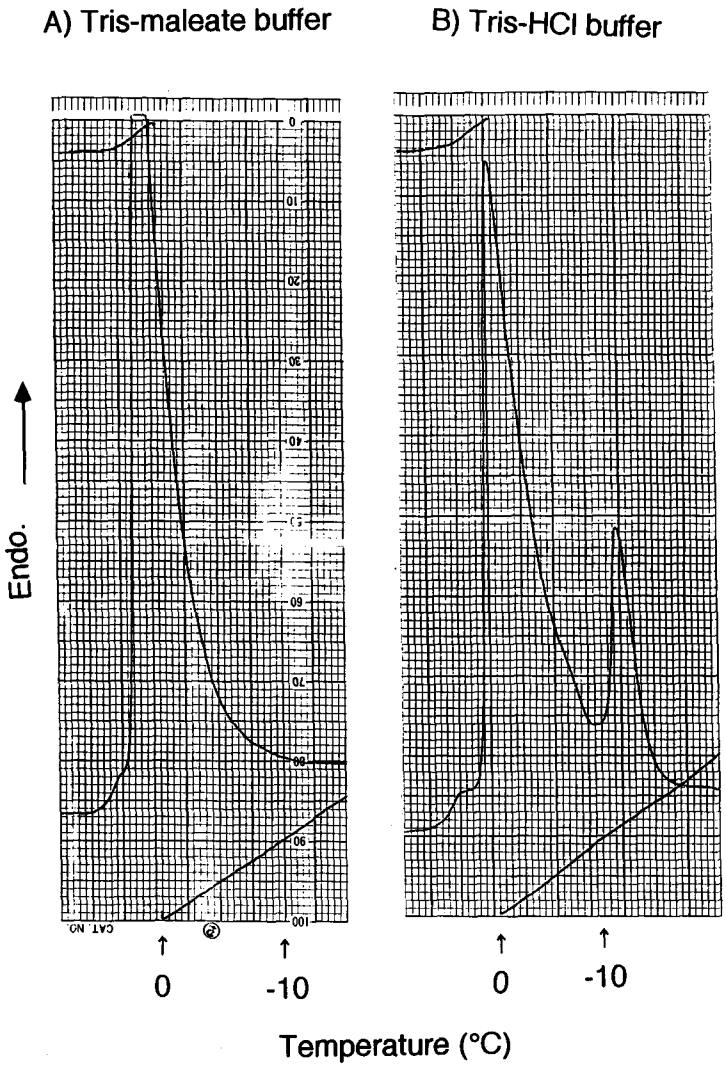

Fig. 6. Differential scanning calorimetry thermograms of Tris-maleate (A) and Tris-HCl (B) buffers.

ATPase activity shown in Figs. 2-4 were not thought to be due only to the pH changes of the buffers (Fennema, 1973b).

In this experiment at −26°C, hardly any change in solubility was observed, except when the 5 mM Tri-HCl buffer was used. And also no noticeable decrease in ATPase activity was observed at −26°C, except when the 5 mM Tris-maleate buffer was used. Since the storage temperature was below the eutectic points of the Tris-maleate and phosphate buffers, and below the Tris saturation point of the Tris-HCl buffer, it was assumed that this phenomenon was due to the smaller amount of unfrozen solution (Takahashi et al., 1993) and the reduction in mobility

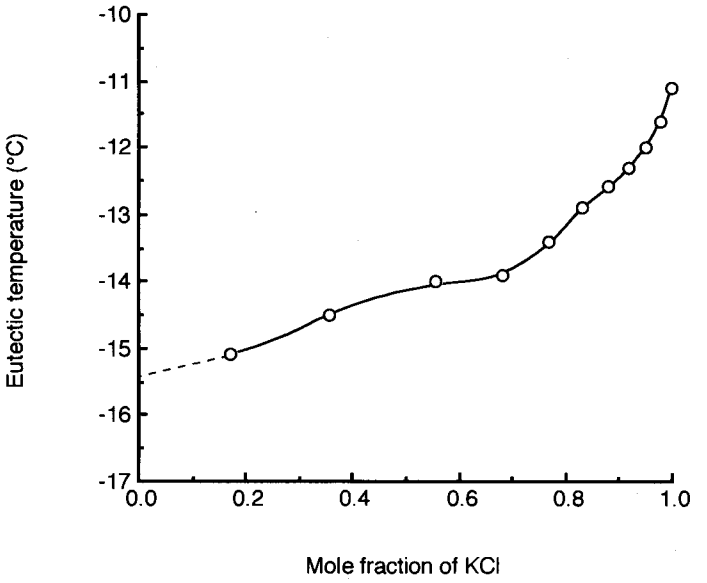

Fig. 7. The plots of the thawing point of phosphate buffer with different mole fractions of KCl. The eutectic point of phosphate buffer without KCl was estimated by extrapolating the thawing points of the solutions to zero of the mole fraction of KCl.

of water molecules in unfrozen solution (Inoue et al., 1996).

The decreases in the solubility and ATPase activity were larger with an increase in storage temperature, except in the following two cases ; the solubility with 50 mM Tris-maleate buffer and the ATPase activity with 5 mM Tris-maleate buffer (Figs. 2, 3, and 4). These results concurred with those reported by Kitazawa et al. (1995) using a phosphate buffer. Comparing the three kinds of buffer systems under 5 mM conditions, the changes in solubility agreed comparatively with the changes in ATPase activity. This could be ascribed to the weak buffer action.

In the case of the 50 mM concentrations, the changes in both indices differed among buffers at -4 and -11°C. The extent of the decrease in solubility and in ATPase activity was smallest with Tris-maleate buffer and phosphate buffer, respectively. No acceptable explanation for these findings have yet been provided. In the case of Tris-HCl buffer, both the indices of solubility and ATPase activity decreased remarkably during the 3 weeks of storage. One of the reasons for the results under this condition may be ascribed to the weak buffer action in an acidic pH range. As for the Tris-maleate buffer, hardly any decrease in solubility was observed, but ATPase activity decreased markedly. A possible explanation for this result is that maleic acid is one of the carboxylic acids which has a preventive effect on the freeze denaturation of myofibrils (Noguchi and Matsumoto, 1970, Ooizumi et al., 1984). In contrast, hardly any decrease in ATPase activity was observed with the phosphate buffer, although the solubility decreased.

The decrease in solubility prior to ATPase inactivation was observed in the 5 and 50 mM phosphate buffers, and a similar relationship in freeze denaturation was reported in the study using real fish flesh (Connell, 1960). On the basis of these

results, the findings that ATPase activity decreased in 50 mM Tris-maleate buffer, while still retaining its solubility, differed from that observed in fish flesh. Takahashi et al. (1993) reported a similar relationship in both indices with 0.1 M KCl and NaCl.

Comparing the effects of the two buffer concentrations on solubility in three different buffers, the extent of decrease in the 5 mM buffer was greater than that in the 50 mM buffer in all cases. This phenomenon could be ascribed to the prevention of freeze denaturation due to an increase in the amount of unfrozen solution in the higher buffer concentration at the same storage temperature. On the other hand, a greater decrease in solubility was found when the storage temperature was higher under almost all conditions studied, except in the case of 50 mM Tris-maleate buffer. Since the amount of unfrozen solution was larger at higher storage temperatures (Takahashi et al. 1993), it is assumed that the extent of the decrease in solubility would be smaller under similar temperature conditions. However, the results were opposite as described above. A possible explanation for this result is that the mobility of water molecules in unfrozen solution may increase at higher storage temperatures [the mobility of water molecules (the apparent dielectric relaxation time) plotted against temperature examined was changed in the logarithmic scale (Inoue et al., 1996)].

Since the extent of ATPase inactivation decreased under all conditions studied with lower storage temperatures, it is assumed that the mobility of water molecules in unfrozen solution was strongly related to the inactivation. In the case of Tris-HCl buffer, no preventive effect on ATPase inactivation was observed at -4 and -11°C when the buffer concentration became higher (Figs. 3A and 4A), although the reason for this is not clear.

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