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Studies on the Freeze Denaturation of Squid Actomyosin*1

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Denaturation of squid actomyosin during frozen storage was studied by measuring solubility, viscosity, and ATPase activity, and by ultracentrifugal analysis and SDS-electrophoresis. The additive effect of sodium glutamate, well-known for its cryoprotective effect on the freeze denaturation of carp actomyosin, was also checked. When solutions (0.6 M KCl) or suspensions (0.05 M KCl) of the isolated squid actomyosin were stored at −20°C, the solubility, reduced viscosity and ATPase activity decreased with the length of frozen storage. The ultracentrifugal patterns showed that aggregation proceeded during frozen storage. Evidently, sodium glutamate prevented the freeze denaturation of squid actomyosin, as in the case of carp actomyosin. When the mantle muscle of squid was freeze-stored at −20°C and extracted with 0.6 M KCl, the amount of soluble actomyosin extractable from the frozen meat and the ATPase activity of the actomyosin were decreased only slightly even after a long freezing period. This differed from the results with isolated actomyosin.

Reflecting the importance of the keeping quality of frozen fish, a great number of studies have been published so far on the freeze denaturation of fish muscle proteins as reviewed by MATSUMOTO.[1] Cryoprotective effects of various compounds on the fish muscle proteins have also been studied,[1] that of L-sodium glutamate being most outstanding as found by the authors' colleagues.[1–3] A comparative review of the above works has led them to propose a unique hypothesis which explains both the freeze denaturation of the fish muscle proteins and the effects of the cryoprotective agents.[4]

Squid meat which has been popularly accepted as a food source by a limited number of nations provides a novel animal protein source for the world.[5] Knowledge about the behavior of the squid muscle proteins during frozen storage seems essential to more extensive development and utilization of this material.

This paper describes our study on the freeze denaturation of actomyosin from the squid mantle muscle, a representative obliquely striated mollusc muscle. Freeze stored squid actomyosin and actomyosin extracted from the freeze stored squid muscle were both studied. At the same time, the effects of L-sodium glutamate, which is known to prevent the freeze denaturation of fish actomyosin, was studied.

Meterial and Methods

Material

Fresh squid *Ommastrephes sloani pacificus* was purchased at the Tokyo Wholesale Fish Market.

Squid Actomyosin

Squid mantle muscle was skinned on both sides, cut into 3 mm cubes and subjected to the extraction and fractionation procedure illustrated in Fig. 1. The obtained actomyosin, P_3, was either dissolved into 0.6 M KCl solution or suspended in 0.05 M KCl solution. The resultant preparations were referred to as actomyosin solution (in high ionic strength) and actomyosin suspension (in low ionic strength), respectively, and subjected to the frozen storage and other experiments. The actomyosin preparation might be more precisely referred to as "paramyosin-containing myosin B" which contains actin, myosin, tropomyosin, troponin and paramyosin.

In vitro Freezing Experiment

Five cm^3 aliquots of the sample solution or suspension adjusted to a protein concentration of about 5 mg cm^{-3} were dispensed into tubes, frozen at −20°C and stored for several terms. To some of the tubes sodium glutamate was added to 0.2 M immediately before freezing. After a definite

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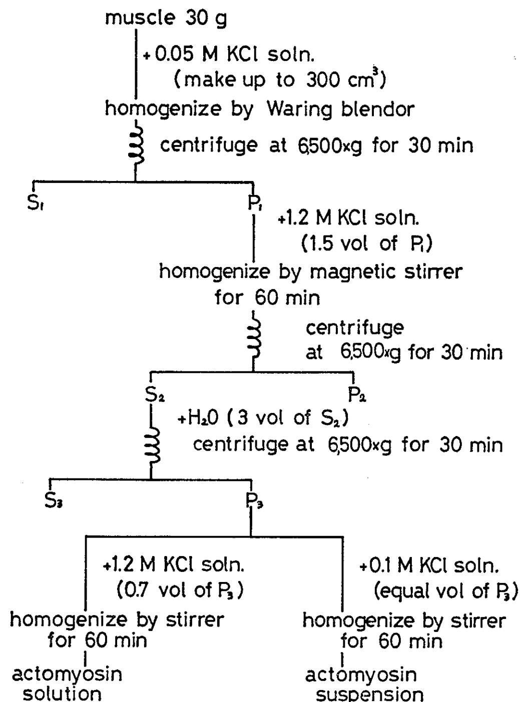

Fig. 1. Preparation of squid actomyosin.

storage period, the sample was thawed at 4°C. homogenized (in case of the suspension sample, with an equal volume of 1.2 M KCl solution), and centrifuged for 60 min at 3,000 rpm. The supernatant, containing the soluble proteins, was submitted to protein determination, viscometry, sedimentation analysis, ATPase activity assay and SDS-electrophoresis, respectively.

In situ Freezing Experiment

The mantle muscle was peeled off the epidermis and each 40 g piece was sealed in a polyethylene bag and freeze stored at −20°C. For storage with sodium glutamate, the muscle was powdered with 10% (*w*/*w*) glutamate prior to freezing.

Protein Determination

The improved biuret method of UMEMOTO was used.[6]

Viscosity Measurement

The viscosity of the protein solutions, diluted to 2 mg cm^{-3} with 0.6 M KCl, was measured with an Ostwald type Viscometer at 25°C.

ATPase Activity Assay

The activity was measured at 25°C under one of the following conditions:

(a) Mg^{2+}-ATPase activity: Protein, 0.2 mg cm^{-3}; Tris-maleate buffer (pH 6.8), 35 mM; ATP, 1 mM; $MgCl_2$, 1 mM; KCl, 50 mM.

(b) Ca^{2+}-ATPase activity: Protein, 0.2 mg cm^{-3}; Tris-maleate buffer (pH 6.8), 35 mM; ATP, 1 mM; $CaCl_2$, 1 mM; KCl, 0.6 M.

Activity was indicated as the millimole amount of the phosphate liberated by a milligram of enzyme protein per minute, the inorganic phosphate determined by FISKE-SUBBAROW's method.[7]

Sedimentation Analysis

The supernatant was ultracentrifuged at speeds ranging from 30,000–45,000 rpm at 20°C in a Phywe Analytical Ultracentrifuge UL 50.

SDS-Electrophoresis

The supernatant sample was treated with SDS reagent at 100°C for 3 min and electrophoresed on 10% polyacrylamide gel as described by WEBER & OSBORN.[8] The densitometry of the gels was performed on a digital computing densitometer, Gelman DCD-16.

Results

Changes of Actomyosin during in vitro Frozen Storage

The squid actomyosin solution in 0.6 M KCl or the suspension in 0.05 M KCl was freeze stored at −20°C and the test tubes were thawed at intervals for analyses and measurements. Some changes were found in the appearance of the thawed solutions stored without sodium glutamate. Namely, a flocculent mass of the proteins was found in the center of the tubes leaving a transparent liquid in the rest of the space. On the contrary, the tubes

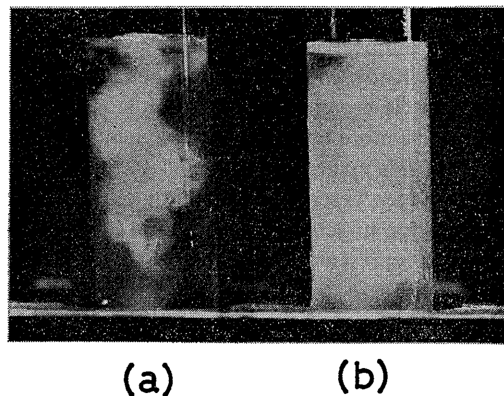

Fig. 2. Squid actomyosin solution, thawed at 4°C for 3 hours after 40 day frozen storage at −20°C with or without 0.2 M sodium glutamate. (a) Stored without glutamate; (b) Stored with glutamate.

with added sodium glutamate did not show any changes in gross appearance and the thawed solutions were always homogeneous throughout the 40 day storage period (Fig. 2). Similar results were found in the case of the suspension samples.

The solubility of actomyosin, namely the amount of the protein remaining soluble in the supernatant of the centrifuged thawed solution, decreased in both cases (Fig. 3). The rate of decrease with time was faster for the suspensions than for the solutions. In the 0.6 M KCl solutions, the sodium glutamate added group gave solubility values slightly higher than those for groups without glutamate added. The effect of the glutamate in preventing the decrease in solubility was outstanding in the case of suspension in 0.05 M KCl.

As shown in Fig. 4, the reduced viscosity values of the soluble proteins of the groups stored without glutamate decreased with the length of frozen storage. The decrease was faster for the suspensions. Here also, these changes were prevented by

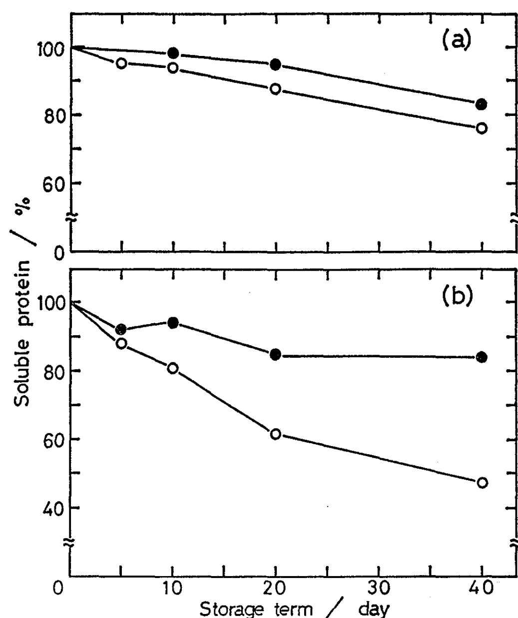

Fig. 3. Changes in solubility of squid actomyosin during frozen storage at $-20°C$ in (a) 0.6 M KCl and (b) 0.05 M KCl with or without 0.2 M sodium glutamate. Values are relative to those of the original unfrozen samples. The concentrations of the original samples were (a) 6.03 and (b) $5.64\,mg\,cm^{-3}$. ○—○, without glutamate; ●—●, with glutamate.

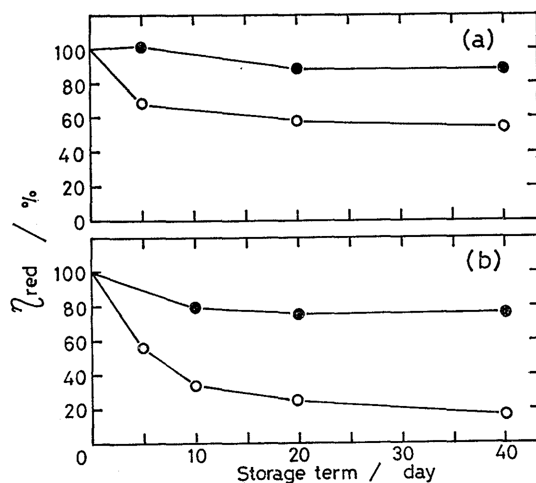

Fig. 4. Changes in reduced viscosity of squid actomyosin during frozen storage at $-20°C$ in (a) 0.6 M KCl and (b) 0.05 M KCl with or without 0.2 M sodium glutamate. Values are relative to those of the original unfrozen samples. The viscosity of the original samples were (a) 0.17 and (b) $0.42\,mg^{-1}\,cm^3$. ○—○, without glutamate; ●—●, with glutamate.

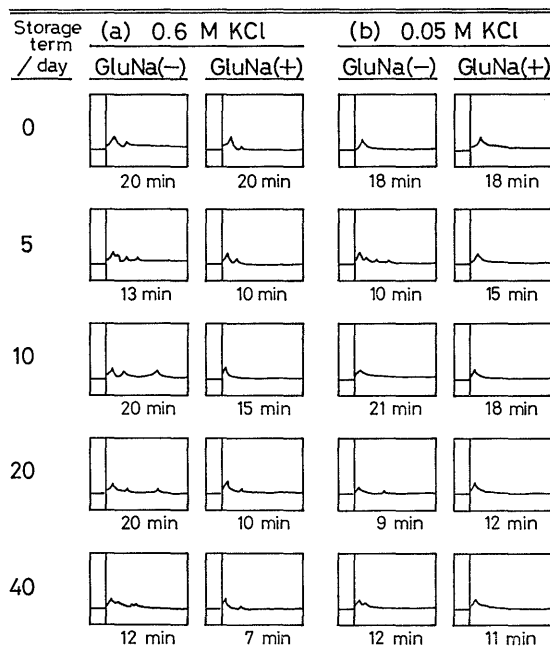

Fig. 5. Changes in ultracentrifugal patterns of squid actomyosin during frozen storage at $-20°C$ in (a) 0.6 M KCl and (b) 0.05 M KCl with or without 0.2 M sodium glutamate. Figures at the bottom of each pattern are the time after reaching 45,000 rpm. The speeds of sedimentation of actomyosin were not equal to one another among the different storage conditions, owing to the difference in the concentration of the sample. GluNa (−), without glutamate; GluNa (+), with glutamate.

the addition of sodium glutamate.

The ultracentrifugal patterns of actomyosin solutions before and after freezing are illustrated in Fig. 5. The original actomyosin sample showed a major peak, 4.3 S, preceded by a small minor peak, 14.7 S (Fig. 5-a). After 5 day storage without glutamate, several small peaks with higher sedimentation coefficients (16 S, 29 S) appeared. After storage for 10 and 40 days, the faster peaks increased in number and in the sedimentation velocity value, while the original major peak became less marked. No significant changes were found in the samples freeze stored with glutamate. Judging from the sedimentation coefficient values, the minor peak, 14.7 S, is most probably assignable to actomyosin, and the major peak, 4.3 S, might include myosin, actin, paramyosin and tropomyosin. The faster peaks appearing after frozen storage must be aggregates and/or conjugates of these proteins.

The dominant occurrence of the 4.3 S peak in the intact actomyosin preparation of squid has been frequently observed in our laboratory and seems to be characteristic of squid actomyosin. A similar anomalous feature has been found in the free phase electrophoreses of the squid actomyosin preparations which otherwise behave as actomyosin.[9] The actomyosin preparation of squid showed the characteristic properties with respect to solubility, viscosity, drop in viscosity upon addition of ATP, ATPase activity, and superprecipitation.[10,11] These features might be explained by postulating that the squid actomyosin is dissociated into actin and myosin when it exists in the dissolved state at high ionic strength. The present 4.3 S peak which is not very sharp might be a combination of the peaks of actin, myosin and paramyosin.

Similar results were obtained in the ultracentrifugal analyses of the actomyosin suspension, which are shown in Fig. 5-b.

The changes in Mg^{2+}- and Ca^{2+}-ATPase activi-

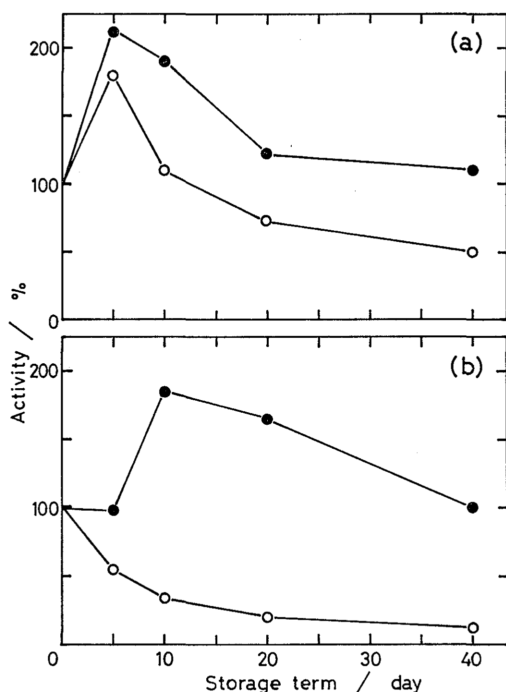

Fig. 6. Changes in Mg^{2+}-ATPase activity of squid actomyosin during frozen storage at $-20°C$ in (a) 0.6 M KCl and (b) 0.05 M KCl with or without 0.2 M sodium glutamate. Values are relative to those of the original unfrozen samples. The specific activity of each original sample was (a) 0.28 and (b) 0.55 μmol $min^{-1} mg^{-1}$. ○—○, without glutamate; ●—●, with glutamate.

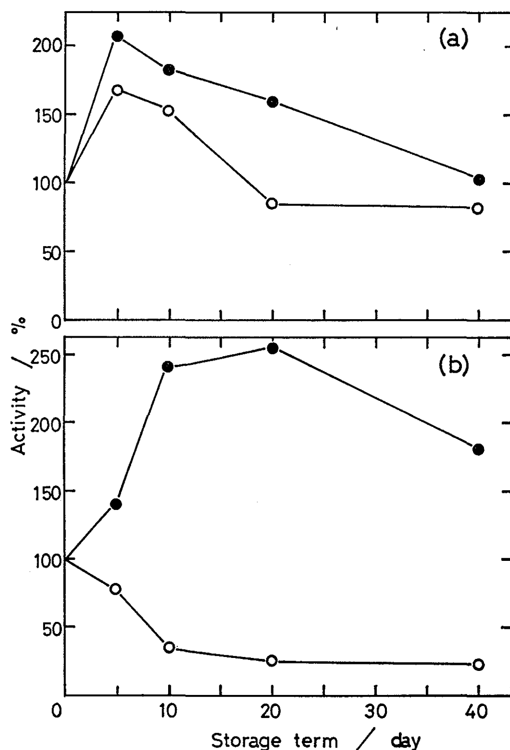

Fig. 7. Changes in Ca^{2+}-ATPase activity of squid actomyosin during frozen storage at $-20°C$ in (a) 0.6 M KCl and (b) 0.05 M KCl with or without 0.2 M sodium glutamate. Values are relative to those of the original unfrozen samples. The specific activity of each original sample was (a) 0.28 and (b) 0.33 μmol $min^{-1} mg^{-1}$. ○—○, without glutamate; ●—●, with glutamate.

ties are illustrated in Figs. 6 and 7. In the actomyosin suspension without glutamate, Mg^{2+}-ATPase activity decreased nearly by a half after 10 day frozen storage, while with glutamate it increased to 200–250% and then decreased but not beyond 50% of the original value (Fig. 6-b). The Ca^{2+}-ATPase activity of the suspension with glutamate showed the maximum activity after 10–20 day frozen storage (Fig. 7-b).

The ATPase activities of the actomyosin solution changed similarly (Figs. 6-a and 7-a), but the rate of the inactivation was less than that of the suspension, 70–80% activity remaining after 40 day frozen storage without glutamate. The increase of the activity after 5 day was also found here in the sample without glutamate.

SDS-electrophoresis was carried out on each supernatant specimen of the above frozen storage experiments. The original solution of squid actomyosin demonstrated an electrophoretic pattern as shown in Fig. 8, in which myosin heavy chain and light chains, paramyosin, actin, tropomyosin, regulatory proteins, and the squid specific 53,000 component were distinguished besides several unidentifiable components.[12] The relative amounts of these components in the freeze stored solutions and suspensions were determined by densitometry and were plotted against the storage period resulting in Figs. 9 and 10. Since each band fraction of the gel appeared to contain not only the proteins primarily native to that protein fraction but also fragments and/or aggregates of proteins deriving from other fractions, a simple comparison is difficult. The 53,000 component and unidentifiable fraction seem to be freeze-resistant since their amounts did not change much. However, in the densitometric traces, they produced broad peaks with several small summits suggesting denaturation taking place.

As for the suspension sample, the largest variation in the amount of each fraction occurred in the

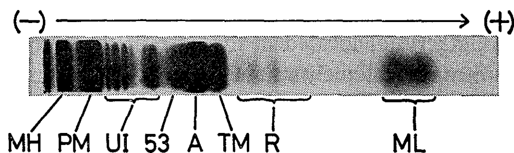

Fig. 8. Electrophoretic pattern of fresh squid actomyosin. Each fraction was assigned as follows: MH, myosin heavy chain; PM, paramyosin; UI, unidentifiable fraction; 53, 53,000 component; A, actin; TM, tropomyosin; R, regulatory proteins; ML, myosin light chains.

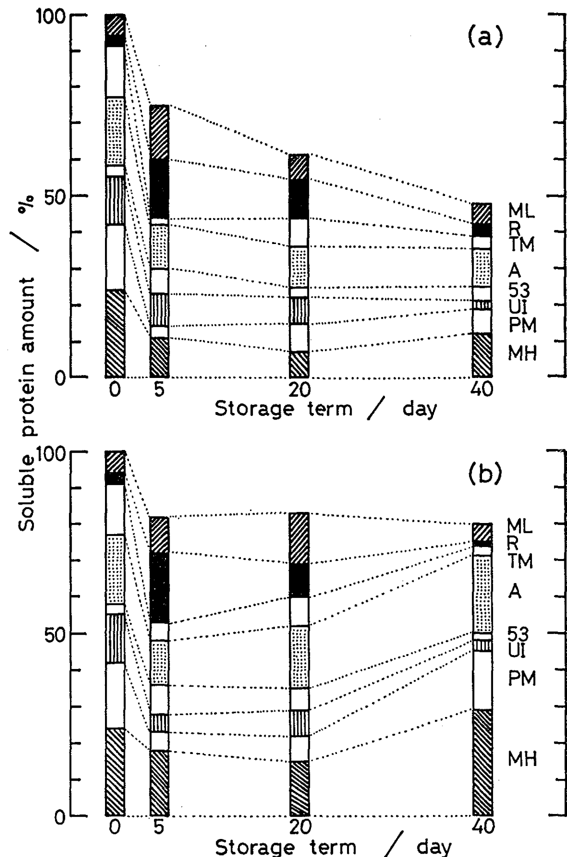

Fig. 9. Changes in amount of each protein fraction in squid actomyosin during frozen storage at −20°C in 0.05 M KCl with (b) or without (a) 0.2 M sodium glutamate. Values are relative to the total protein amount of the original unfrozen actomyosin suspension. ML, myosin light chains; R, regulatory proteins; TM, tropomyosin; A, actin; 53, 53,000 component; UI, unidentifiable fraction; PM, paramyosin; MH, myosin heavy chain.

first 5 days of storage. Myosin light chains and regulatory proteins increased in proportion to the decrease of myosin heavy chain+paramyosin+tropomyosin. This suggests that the large polymers were broken down into small fragments. The amount of actin changed little during frozen storage. Generally, fibrous proteins appear to be more apt to freeze-denature as compared to globular ones.

In the results obtained with the actomyosin solution (Fig. 10), the amount of myosin heavy chain, paramyosin, and myosin light chains decreased. The changes in amounts of actin, 53,000 component and the unidentifiable fraction were

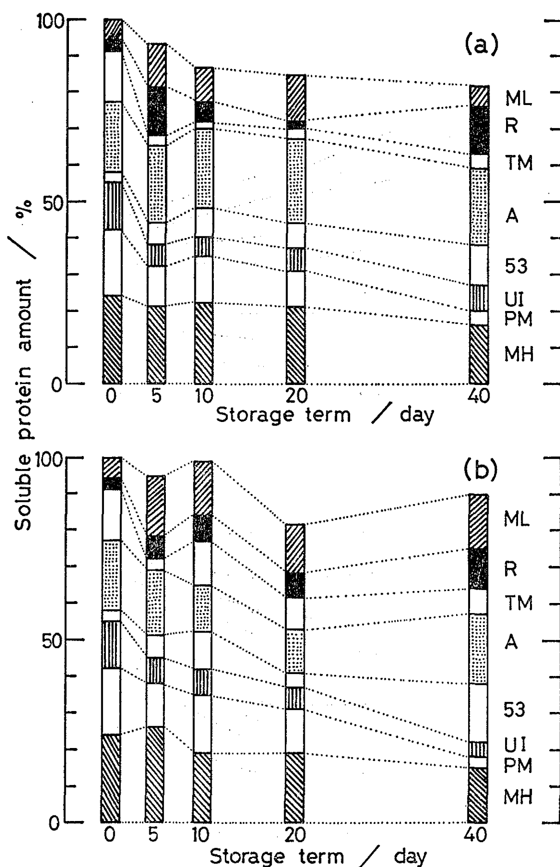

Fig. 10. Changes in amount of each protein fraction in squid actomyosin during frozen storage at −20°C in 0.6 M KCl with (b) or without (a) 0.2 M sodium glutamate. Values are relative to the total protein amount of the original unfrozen actomyosin solution. ML, myosin light chains; R, regulatory proteins; TM, tropomyosin; A, actin; 53, 53,000 component; UI, unidentifiable fraction; PM, paramyosin; MH, myosin heavy chain.

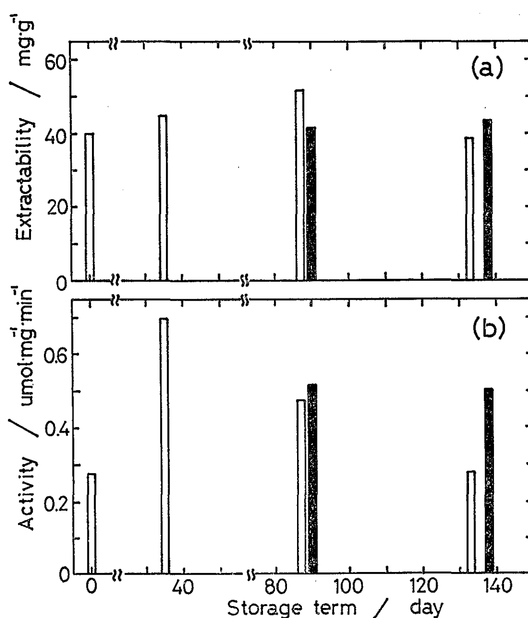

Fig. 11. Changes in extractability of actomyosin (a) and Ca^{2+}-ATPase activity of the extracted actomyosin (b) of squid mantle muscle during the in situ frozen storage at −20°C with or without 10% (w/w) sodium glutamate powdered. The extractability was described as the soluble actomyosin amount extracted from 1 g meat. ▭, without glutamate; ■, with glutamate.

small. The additive effects of glutamate were not apparent in either case.

Changes in Actomyosin during in situ Frozen Storage

Pieces of the squid mantle muscle were freeze stored with or without sodium glutamate added and were submitted to extraction with 0.6 M KCl at intervals during storage over a period of 100 days. As shown in Fig. 11, no tendency, either to decrease or to increase, was found throughout the storage period studied. Effect of the added glutamate was inappreciable. The ATPase activity first increased and then decreased (Fig. 11-b) as in the case of the in vitro freezing experiment.

Discussion

The present results on the frozen storage of squid actomyosin, which demonstrates a decrease in solubility and viscosity during frozen storage, indicate that denaturation occurs. The profiles of the decreases in these properties were similar to those of carp actomyosin, but the rate of the decrease appeared slower than with carp actomyosin.[2,13,14] Considering this rate-difference in relation to the difference between skeletal muscle and obliquely striated muscle, the involvement of paramyosin in squid actomyosin is suggested. Paramyosin filaments which do not exist in skeletal muscle form the paracrystal located in the core of the myosin filaments of obliquely striated muscle. It is known that myosin is more susceptible to frozen storage than actin, and the function of paramyosin may be to support the myosin filaments thus preventing their unfolding, decomposition and aggregation.

The decrease of solubility indicates that inter- and/or intra-molecular bonds were formed and the filaments intertwined. This caused aggregation and insolubilization of actomyosin. As the solu-

bility was preserved better in 0.6 M KCl solution than in 0.05 M KCl suspension, ionic bonds are thought to be one of the cross-linking bonds which cause aggregation.

The results of viscometric and ultracentrifugal data show that the size and shape of the proteins also changed. The fall in viscosity suggests that the actomyosin filaments have either assumed a less asymmetric shape or have been cut into shorter filaments and fragments. As discussed before, there are some ambiguities in the assignment of the ultracentrifugal peaks for the intact actomyosin solution. Nevertheless, it is by all means probable that aggregation of the proteins took place during the frozen storage. Some aggregate peaks appeared after short storage, but they were not observed after long storage, probably because these aggregates eventually became insoluble and thus could be removed by centrifugation after thawing.

The increase of ATPase activity after a short frozen storage term was probably caused by wobble of the molecular conformation, as has been suggested for carp actomyosin, myosin, and heavy meromyosin.[4]

The results of SDS-electrophoresis suggest that myosin, paramyosin, tropomyosin, and regulatory proteins denatured much more quickly than actin, though, as described in Results, it is very difficult to distinguish and determine the fraction of each protein. Better methods for studying intramolecular denaturation are desirable. Electrophoresis was also tried on the precipitate insolubilized during frozen storage, but broadening and marked tailing of the bands were observed. There might have been such a large amount of aggregates that no more could be dissociated by SDS treatment.

At least two types of denaturation might have occurred during frozen storage. One is the aggregation of the protein molecules as suggested by the decrease in solubility and the results of sedimentation analysis. The other is the fragmentation and conformational changes of the protein molecules, as indicated by the electrophoretic data.

The preventive effect of sodium glutamate against the freeze denaturation of the squid actomyosin was clearly demonstrated in every aspect of the study, in accord with the data on actomyosin and myosin of carp.[2] It was observed that the sample with glutamate was highly homogeneous even after frozen storage and thawing as shown in Fig. 2. Glutamate must work to promote the water-affinity (or solvent-affinity) of the protein molecules and/or to prevent water from freezing and crystallizing near the tube wall thereby concentrating the proteins and other solutes in the center. Thus, in the presence of glutamate, the protein molecules would be maintained at some distance apart from each other hindering aggregation. The pressure caused by the volume increase of the crystallizing water surrounding the protein molecules would be reduced, thus preventing damage of the protein molecules and/or acceleration of aggregation. This could explain the observation of homogeneity in the samples with glutamate. The mechanism of the cryoprotective effects of sodium glutamate is discussed in detail elsewhere.[1,2,4]

The data on the frozen storage of the squid muscle illustrated that the proteins of intact muscle undergo much less change as compared with proteins extracted and freeze stored in the state of solution or suspension. Though this is also true in the case of fish muscle proteins, the squid proteins appear to be more resistant against freeze denaturation in situ. The profiles of the in vitro freeze experiment may not be precisely equal to the protein denaturation profiles during in situ frozen storage, but rather represent the overall tendency involved in the latter.

On the other hand, the squid muscle proteins are characteristically labile when they are kept in situ or in vitro near the freezing point or at room temperature.[9,11] This instability has been attributed to the lability of the protein molecules of squid and/or to the effects of the proteinases existing in the muscle.[9,11,15]

The high stability of the squid muscle proteins against freeze denaturation as illustrated here is interesting in view of the above mentioned instability during cold storage. The fact that the preparation of actomyosin samples from once-frozen muscle is less difficult than from the fresh muscle might be due to the inactivation and/or denaturation of the proteinases by the frozen storage.

The changes in ATPase activity of the actomyosin extracted from frozen muscle suggests that wobble of the active site of the molecule might also have occurred here in situ during frozen storage, as has been suggested to occur during in vitro storage.[4]

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