Studies on the Control of the Denaturation of the Fish Muscle Proteins during the Frozen Storage—I. Preventive Effect of Na-glutamate*

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For the further improvement of the frozen storage of meat and fish meat, deeper knowledge of the mechanism of the freezing denaturation of the muscle proteins as well as better techniques controlling the denaturation are needed. With respect to the denaturation mechanism, various theories or hypotheses have been presented so far, attributing its main cause to the aggregation of the protein molecules¹⁾, either through the removal of water^{2,3)}, or through the salting-out of the proteins⁴⁾, to the poisonous effect of the free fatty acids⁵⁾, to the pH change⁶⁾, to the formation of formaldehyde⁷⁾, etc., but the complicated process of the denaturation brings about difficulties in studying the methods of its control.

With a view to obtain information on the denaturation mechanism through the interaction of the proteins with other substances, as well as to find out some new materials to prevent the denaturation, the authors have been testing the denaturation of a frozen actomyosin preparation of the carp at the presence of a variety of additives, and have found out a preventive effect of monosodium-glutamate (Na-glutamate).

The present paper deals with the *in vitro* model tests for the effect of Na-glutamate on the isolated actomyosin preparation and also with the study on the quality of the "Kamaboko" jelly which is processed from the minced fish meat frozen-stored with and without added Na-glutamate. The latter experiment was performed to check the applicability of adding Na-glutamate to the Reitosurimi (frozen minced fish meat). Recently, the Reitosurimi became the major material in the Kamaboko manufacture. Moreover, it was aimed that the results with the jelly forming ability as revealed in the quality of Kamaboko could make an information of the wider applicability of this method to the control of the frozen storage denaturation of other foods such as meats and meat products, because the jelly-forming ability of a material is a good index of the state of the actomyosin molecules which makes the major factor of the texture of meats.

The tests in 0.05 M KCl make a model for the frozen storage of the meat and the

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salt-free minced fish meat, while those in 0.6 M KCl do so for that of the salt-added minced fish meat.

Methods

1. Materials.

Actomyosin preparation: Carp (koi), *Cyprinus carpio*, ca. 30 cm long, was killed by decapitation and its dorsal lateral muscle was cut out immediately, being subjected to the extraction and fractionation as illustrated in Fig. 1. The resultant actomyosin was either dissolved into 0.6 M KCl solution or brought in suspension in 0.05 M KCl for the model tests.

Minced fish meat for Kamaboko jelly test: Muscles of the fresh horse mackerel (maaji), *Trachurus japonicus*, purchased at the Tokyo Central Fish Market, have been cut out, minced with a chopper and washed with tap water for several times, and used for the Kamaboko jelly tests.

2. Model test of freezing denaturation (*in vitro* model test).

Operation of the test: To the actomyosin solution in 0.6 M KCl, a calculated amount of the solution of Na-glutamate, glucose or urea dissolved in 0.6 M KCl was added to obtain the needed final concentration of the additive and mixed with a glass-stirrer.

In the case with the experiments at 0.05 M KCl, the above actomyosin suspension in 0.05 M KCl was used. The final concentrations in the test mixtures were 0.3 M for Na-glutamate, 1.0 M for glucose and 0.05 M for urea, respectively, in both the experiments at 0.6 M and 0.05 M KCl. The protein concentration in the test mixtures ranged from 2.5 to 4 mg protein/ml.

Each 10 ml of the mixtures was put in a test tube, stoppered, frozen and stored at -20°C for various periods. For measuring the rate of denaturation, the mixture in each test tube is taken out at intervals, thawed at the room temperature, homogenized in the tube, and subjected to determination of the solubility, viscosity, and sedimentation, respectively.

Solubility: The homogenized thawed solution was centrifuged for 20 min at 3,000

Fig. 1. Preparation of actomyosin for the *in vitro* model test.

rpm and at °C, the protein concentration of the supernatant being determined from the absorbance at 275 m μ , by using a conversion factor, 6.25×0.12 mg protein/ml/Absorbance (cm).

In the case with the tubes of 0.05 M KCl, an equal amount of 1.2 M KCl solution was added to the thawed suspension before homogenizing, to bring the salt concentration to 0.6 M KCl.

Viscosity: A portion of the supernatant was taken and diluted with 0.6 M KCl into a series of the solutions of different protein concentrations. The viscosity of these solutions was measured with OSTWALD's viscometer at 25°C, and the relative viscosity for 2 mg protein/ml obtained by the interpolation of the data was adopted. This operation was done in place of using the intrinsic viscosity because the viscosity of the fish actomyosin does not always follow the linear function with the protein concentration especially at the region of very low protein concentration⁸⁾.

Sedimentation: A few ml of the supernatant was ultracentrifuged at speeds ranging from 30,000 rpm to 50,000 rpm at 20°C, by the use of PHYWE Ultracentrifuge UL50.

ATPase activity: For measuring the effect of the frozen-storage on the ATPase activity, the similar frozen-storage test of the actomyosin solution or suspension was performed on a smaller scale of 1 ml in each tube. The whole protein suspension, without the centrifuging process, was used for measuring the ATPase activity.

When the mixture in the tube was thawed, 1.0 ml of 0.24 M tris-malate buffer, 1.6 ml of 1.7 M KCl (2.1 M KCl in the case with the sample tube of 0.05 M KCl), 0.4 ml of 10 mM CaCl₂, and finally, 2 ml of 2.2 mM Na-ATP solution were added in turn and the rate of release of the inorganic phosphate from the added ATP was determined by FISKE-SUBBAROW's method⁹⁾ after stopping the reaction by adding trichloroacetic acid. The final concentration of the components in the reaction mixture was, ATP 2.2 mM/l, CaCl₂ 0.7 mM/l, tris-malate buffer (pH 6.3) 0.04 M/l, KCl 0.56 M/l: The contribution to the ionic strength of the additives was negligibly small.

3. Kamaboko-jelly test.

To 300 g of the above washed mince of the horse mackerel meat, 3.74% (w/w) Na-glutamate was added, ground for 10 min in the mechanical mortar, packed in polyethylene-cellophane-film bag, and frozen-stored at -20°C for one and half month. Another lot of the washed mince without added Na-glutamate, as the control, was packed and stored in the same manner.

For preparing the Kamaboko jelly, each frozen block was thawed at room temperature, added with 2.5% of NaCl and 0.2% of Na-polyphosphate, ground in a mechanical mortar for 30 min, packed into polyvinylidenechloride casing, sealed, boiled for 50 min at 85°C, and subjected to the jelly tests after stored overnight in the refrigerator according to OKADA's method¹⁰⁾. Na-pyrophosphate was added to increase the elasticity of Ka-

maboko jelly.

Quality of Kamoboko jelly: OKADA's gelometer was used for measuring the jelly strength¹⁰⁾, and the expressible water was determined under a pressure, 10 kg/cm² by using a hand press¹¹⁾. The folding test was applied according to OKADA, defining the classes of the quality by the grade of the durability of the jelly, when its disc (3 cm in diameter, 5 mm thick) was folded once into a semicircle or twice into a quadrant as follows:

- AA, No crack when folded into a quadrant,
- A, No crack when folded into a semicircle,
- B, Cracks when folded into a semicircle,
- C, Breaks into two pieces when folded into a semicircle.

Solubility: 10 g of the thawed meat was homogenized with 190 ml of 0.6 M KCl for 2 min by using a baffle-plated Waring-blendor, succeeded by centrifuging for 20 min at 3,000 rpm. The protein concentration of the supernatant was determined by the biuret method¹²⁾.

Results

Frozen-storage in 0.6 M KCl. As shown in Fig. 2-a, the solubility of actomyosin of the groups added with 0.3 Na-glutamate or 1.0 M glucose did not fall during the frozen-storage, while that of the control group fell faster than the above. The urea-added group lost the solubility even faster than the control.

As shown in Fig. 2-b, the viscosity of the group containing Na-glutamate kept a comparable level with the unfrozen material after the storage as long as 10 weeks, whereas the control curve went down quickly. In the glucose-added group, it fell in between the

Fig. 2. Effect of Na-glutamate, glucose and urea on the denaturation of the actomyosin during the frozen storage in 0.6 M KCl at -20°C . a) Solubility, b) Viscosity, c) ATPase activity. In the figures of solubility and ATPase activity, the values show the percentage to those for the fresh actomyosin before the frozen storage.
 —x—: control, —○—: 0.3 M Na-glutamate,
 —●—: 1 M glucose, —△—: 0.05 M urea.

Na-glutamate-added group and the control. These results suggest that the conformation, or the size and shape, of the actomyosin molecules did not undergo much change during the frozen-storage when they were frozen with Na-glutamate.

Fig. 2-c, which illustrates the change of the ATPase activity during the frozen storage for 10 weeks, shows a fall down to 20% at the control group, while the original enzymatic activity was reserved in the Na-glutamate-added group. The line for the glucose-added group showed some depression at the later period and urea seemed of detrimental effect on the ATPase activity during the frozen storage.

Fig. 3. Ultracentrifugal patterns of the actomyosin before and after the frozen storage in 0.6 M KCl at -20°C for 10 weeks with or without added Na-glutamate or glucose. The speeds of sedimentation of actomyosin is not equal to each other among the different conditions, owing to the differences in the concentration of the additives.

The sedimentation patterns as shown in Fig. 3 reveal that the sharp actomyosin peaks of the control were lost after the frozen-storage, while that of the Na-glutamate-added group was still sharp even after 10 weeks. It suggests the preventive effect of Na-glutamate on the denaturation during the frozen-storage. The actomyosin peak of the glucose-added group became broader after the frozen storage. The comparison of the sedimentation velocities of the different conditions is not very significant, because the concentration of the proteins and the additives are not equal to each other.

Frozen storage in 0.05 M KCl. In contrast to the above experiments, actomyosin is in a suspended state in 0.05 M KCl. As shown in Figs. 4 and 5, data of each the solu-

Fig. 4. Effect of Na-glutamate and glucose on the denaturation of the actomyosin during the frozen storage in 0.05 M KCl at -20°C . with the values expressed in percentage, same as Fig. 2.

—x—: control, —○—: 0.3 M Na-glutamate, —●—: 1 M glucose.

Fig. 5. Ultracentrifugal patterns of the actomyosin before and after the frozen storage in 0.05 M KCl at -20°C for 4.5 weeks with or without added Na-glutamate or glucose. The patterns at 5 min after reaching 50,000 rpm are shown. The speed of sedimentation of actomyosin on the pictures of Fig. 5 appears higher than some of those of Fig. 3, probably owing to the different concentrations of the protein and the environment: Test solutions in Fig. 5 are diluted with 1.2 M KCl. (cf. Methods, B.)

bility, viscosity, ATPase activity and sedimentation analysis show that the rate of frozen-storage denaturation was depressed by the addition of Na-glutamate or glucose in generally the same manner as the case found in 0.6 M KCl.

Effect and concentration of Na-glutamate. The preventive effect of various Na-glutamate concentrations on the protein denaturation was studied by using the *in vitro* model test in 0.6 M KCl, resulting in Fig. 6. Among the data ranging from 0.002 M

to 0.5 M of Na-glutamate, the effect was found to approach the maximum beyond 0.025 M (0.42%).

Reproducibility of the solubility and the ATPase activity measurements. Each datum for the solubility and the ATPase activity appearing on Figs. 2 and 4 represent the mean value of the triplicate measurements of the 3 separate tubes. The reproducibility of the measurement was good as is indicated by the values of the maximum coefficient of variation: 3.5% for the solubility both in 0.6 M KCl and 0.05 M KCl, and 1.7% for the ATPase activity in 0.6 M KCl and 3.9% in 0.05 M KCl, respectively. There is no significant difference between the coefficient of variation in 0.6 M KCl and that in 0.05 M KCl, and these results show the precision of the method.

Quality of "Kamaboko jelly" processed from the meat after storage. As shown in Table 1, the meat frozen-stored for 6 weeks with added Na-glutamate produced a Kamaboko jelly which is higher in the jelly strength, less in the expressible water, and better on the folding test, respectively, as compared with the control Kamaboko which was prepared from the meat frozen-stored without added Na-glutamate. Moreover, the latter product consisted not of a smooth jelly of homogeneous texture but of a rough touching mass of heterogeneous one which is conventionally called "Tsumire". Also with the solubility of the proteins of the thawed mince, there was, in parallel to the difference in the qualities of the Kamaboko jelly, a marked difference between the groups with and without added Na-glutamate.

Table 1. Kamaboko jelly forming ability and soluble proteins of fish meat mince frozen-stored at -20°C for 6 weeks with or without added Na-glutamate.

	Jelly strength (g·cm)	Expressible water (%)	Folding test	Soluble proteins (%)*
Na-glutamate added	1,634	26.3	AA	10.5
Control	890	28.8	A(B)	8.0

* for weight of wet mince.

Fig. 6. Relationship between the effect and the concentration of Na-glutamate on freezing denaturation of carp actomyosin in 0.6 M KCl at -20°C for 1 month.

Discussion

The *in vitro* model tests and the Kamaboko jelly tests revealed that 0.3 M Na-glutamate is as effective as 1 M glucose, whose preventive effect on the frozen-storage denaturation is as comparable as other sugars¹³⁾.

The correspondence of the effects of Na-glutamate between in the model test and in the Kamaboko-jelly test suggests that the frozen storage denaturation of actomyosin is prevented by the added Na-glutamate in both cases.

Na-glutamate has been used commonly for improving the flavour of foods and it is also added to the ground paste of fish meat immediately before the last heating process in the Kamaboko manufacture. NORTON *et al.*¹⁴⁾ has reported the protective effect of Na-glutamate on the overall keeping quality of frozen-stored meats and meat products where the antioxidant effect of Na-glutamate is notified, and the preventive effect of Na-glutamate against the damage of freeze-dehydrated BCG^{15,16)} as well as that of the freeze-dehydrated bacteria has been reported¹⁷⁾. However, it is a new finding that Na-glutamate reduces the denaturation of the actomyosin of fish meat protein during the frozen storage. This fact might provide a new technique of the frozen-storage of fish meats and meats including their products.

In recent years, the expansion of the fish consumption and the limited productivity of the off-shore waters have been driving the fishing area farther from the lands where the processing plants and consuming areas are located, and is bringing up keener need for preventing the frozen-storage denaturation of the muscle proteins. In the Japanese Reitosurimi (frozen minced fish meat) industry which supplies the majority of the material of the Kamaboko jelly industry, the concomitant use of the polyphosphates and sucrose as additives is popular at the present¹³⁾. Na-glutamate has some advantage over the polyphosphate in the nutritive point of view, while replacing a portion of the sugar by Na-glutamate decreases the excessive flavour of sugar in the Kamaboko.

The present results also suggest that the testing system which is based on the *in vitro* freezing of the isolated actomyosin or myosin is useful in following the denaturation process for the precision of its results and for the ease and speed-up of the operation. Whereas such system for studying the freezing denaturation has been first adopted by SNOW¹⁸⁾ in his work on the effect of the salts, and also by CONNELL¹⁾ for comparing the effect of freezing velocity and storage temperature, the present study suggests that this model test is applicable to screening the additives for the preventive function to the frozen-storage denaturation. Recently, a similar method has been reported by ARAI *et al.*¹⁹⁾.

As shown in the figures, the results of the experiments carried out in 0.6 M KCl are substantially the same with those in 0.05 M KCl with regards to the effect of the added Na-glutamate and glucose.

SAKAI²¹⁾ has reported the preventive effect of the low concentration urea on the freezing injury of the plant tissues. In our experiment, 0.05M urea did not prevent the frozen-storage denaturation of actomyosin but it promoted the latter, while, as shown in Fig. 2, the actomyosin containing 0.05 M urea showed the same values in solubility, viscosity, and ATPase activity as those of the urea free actomyosin when it is tested before freezing.

Some workers^{22,23)} have also reported that urea of the lower concentration may not induce the denaturation of myosin A, whereas urea of the higher concentration promotes it. On the other hand, OKADA²⁴⁾ found the promotive effect of urea (1 M) on setting phenomenon (Suwari) of fish muscle which seemed to involve a conformational change of the fish actomyosin. On these basis, our results might be reasonably explained by assuming that the urea concentration around the protein molecules is raised as a result of the ice-formation when the solution is frozen.

The mode of frozen-storage denaturation of the carp actomyosin as revealed in the changes of solubility, as well as ATPase activity appears similar to those obtained by CONNELL²⁰⁾ on the extractable proteins from the frozen-stored cod muscle except for the CONNELL's data of the ATPase activity for the storage at -29°C , where no fall was found.

Summary

1. The effect of the monosodium glutamate on the frozen-storage denaturation of the fish muscle actomyosin was studied by an *in vitro* model test of the isolated actomyosin as well as by a frozen-storage test of the washed mince of the fish meat. Besides the Na-glutamate-free control group, glucose-added and urea-added groups were examined for comparison.

2. In the model test, 0.3 M Na-glutamate was found as effective as or even better than 1 M glucose in preventing the denaturation of actomyosin as far as the data on the solubility, viscosity, ATPase activity and the ultracentrifugal patterns are concerned (Figs. 2-5). There was not much difference between the results carried out in 0.6M KCl and those in 0.05 M KCl. The addition of urea was rather promotive to the denaturation.

3. In 0.6 M KCl, the effect of Na-glutamate approached the maximum beyond 0.025 M (0.42%) (Fig. 6).

4. The Kamoboko jelly made of the frozen mince with added Na-glutamate was better than those of the control group in the keeping qualities as measured by the jelly strength, folding test and the amount of expressible water, and also by the amount of soluble proteins (Table 1).

5. The present results suggest the applicability of the model test for screening the additives for preventing the freezing denaturation, as well as for studying its mechanism.

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