ウマヅラハギ筋肉タンパク質の熱ゲル化におよぼす架橋剤の影響

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Effects of Cross-linker on Thermal Gelation of Oval Filefish Muscle Proteins

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Effects of a cross-liker, 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC), on the thermal gelation of oval filefish meat and myosin pastes were examined by means of thermal temperature-jelly strength curves (30°, 40°, 50°, 60°, 70°, and 80°C) and the activities of ATPases. The treatment with 0.5% EDC (w/w) gave higher jelly-strength values to the meat paste for all the tested temperatures. "Himodori" (thermally induced gel disintegration) for meat paste and myosin was completely depressed by EDC. By treating the actomyosin with EDC (0.05 mg EDC/mg protein), the activation of Mg-ATPase and inactivation of EDTA-ATPase occurred simultaneously while Ca-ATPase was unchanged. Acto-S-1 Mg-ATPase was also activated 1.8 to 2 times by EDC treatment. The possible role of the cross-linker, EDC, in the thermal gelation of oval filefish muscle proteins was discussed.

Although an important technological property of protein for muscle-based gel-type foods is gel-forming ability, distinct differences with regard to the ability are observed among fish species. This difference could be explained primarily by the difference in rate and degree of thermal denaturation of myofibrillar proteins, especially myosin. More detailed information on this aspect has, however, not been available as yet.

Recently, it was reported that the formation of cross-linked myosin heavy chains plays an important role in the "setting" of Alaska pollack surimi.[1–3] In our previous papers,[4,5] it was shown that the mechanism of thermal gelation of oval filefish muscle proteins was really complicated, accompanying an gel-disintegration which occurs during heating at 60°C ("Himodori"). The thermal gelation involves generally intramolecular and intermolecular changes in protein. To clarify their mechanism, the study on protein structure accompanying changes in the state of thermal gelation will be required. Very recently, changes in the activity of carp myofibrillar ATPases treated with a chemical cross-linker, 1-ethyl-3-[3(dimethylamino)propyl]carbodiimide (EDC), were reported.*2

To obtain information about thermal gelation of cross-linked protein complexes with EDC, the present paper deals with the effects of EDC on the thermal gelation of oval filefish muscle proteins and the actomyosin ATPases.

Materials and Methods

Materials

Chymotrypsin and phenylmethyl-sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. Ltd. The cross-linker, 1-ethyl-3-[3-(dimethyl amino)propyl]carbodiimide (EDC) used in this experiment was obtained from Wako Chemical Ltd. All other chemicals used were regaent grade. Oval filefish *Navodon modestus* in full rigor was obtained from local retail store.

Sample Preparation

In the preparation of meat paste, the dorsal muscle was minced in a chopper (hole size 3 mm) and the minced meat was washed twice with 10 vol. of 0.3% $NaHCO_3$-0.15% NaCl to remove blood and water soluble components. The washed meat obtained on centrifugation (12,000 × g, 20 min) was adjusted to a moisture content of 84–85% (w/w), and pH 6.8–6.9 by the addition of 0.1 N NaOH, if necessary. The meat and 2.5% NaCl added by weight were ground into a meat paste.

In the preparation of muscle proteins, the dorsal muscle was carefully excised. Myosin was pre-

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*2 K. Konno: Abstract of the Annual Meeting of the Japan. Soc. Sci. Fish., Tokyo, April, p. 241 (1987).

pared from the muscle by the method of Mackie and Connell[6] with some modification. The myosin solution, containing 0.5 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 20 mM tris-maleate buffer (pH 7.5), was clarified by ultracentrifugation at 10,000 × g for 3 h by the method of Weber,[7] and then myosin in the supernatant was collected by fractional precipitation (40~55% ammonium sulfate saturation). For the thermal gelation of myosin, the paste containing 90% moisture and 2.5% NaCl (w/w) was obtained after the myosin suspension in 0.03 M KCl, 10 mM phosphate buffer (pH 6.4~6.5) was centrifuged at 20,000 × g for 30 min. Myosin subfragment-1 (S-1) was prepared according to the method of Weeds and Pope.[8] Proteolysis (chymotrypsin) was terminated by the addition of 0.5 mM PMSF after digesting at 10°C for 15 min. The fragment thus obtained was further purified by Sephadex G-200 gel filtration (2.2 × 88 cm). Actin was obtained from the aceton-dried muscle by the method of Spudich and Watt[9] and transformed into F-actin by polymerization in 0.1 M KCl and 1 mM $MgCl_2$. All procedures above were conducted at 2~5°C.

Jelly-Strength Test

For the cross-linking of protein, to the meat (pH 6.8) and myosin (pH 6.4) pastes was added 5% cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC)solution, to bring finally 0.05~0.5% (w/w). The EDC-pastes were packed in stainless steel tubes (diameter 3.0 cm × height 3.0 cm for meat paste, 2.0 cm × 1.5 cm for myosin paste) and after the cross-linking reaction (18~20°C, 60 min), heated at 30°, 40°, 50°, 60°, 70°, and 80°C for 5, 20, 60, 120 min, respectively, unless otherwise specified. The gels formed in the tubes after heat treatment were cooled in ice water, kept at room temperature, and then subjected to jelly-strength measurement with a rheometer (Type R-UDJ, San Kagaku Co. Ltd., ball type plunger, diameter 0.5 cm). The jelly-strength was expressed as the product of breaking load and breaking strain (g·cm). The gel-disintegration ("Himodori") index was given as the percent of [1-(jelly-strength at 60°C for 120 min/jelly-strength at 50°C for 20 min].[10] Each measurement was made on three to five samples and standard errors of measurements are shown as error bars.

Assay of ATPase Activity

The actomyosin or acto-S-1 was obtained from myosin or S-1 and F-actin in an amout equal to myosin or S-1, respectively. The actomyosin in 0.6 M KCl and acto-S-1 in 0.1 M KCl were treated with varying amounts of EDC (0.002~0.05 mg EDC/mg protein). The cross-linking reaction was allowed to proceed for 60 min at 20°C after the addition of EDC, unless otherwise stated. Their ATPase activities were assayed at 25°C for 2 min after 5 min pre-incubation. The assay mixtures (2 ml) contained actomyosin (0.3 mg/ml) or acto-S-1 (0.1 mg/ml) together with 1 mM $MgCl_2$, 0.03 M KCl 10 mM imidazole (pH 7.0) for Mg-ATPase; 0.6 M KCl, 5 mM EDTA, 10 mM imidazole (pH 7.0) for EDTA-ATPase; and 0.05 M KCl, 5 mM $CaCl_2$, 10 mM imidazole (pH 7.0) for Ca-ATPase, respectively. The reaction was started by the addition of 2 mM ATP for Mg-ATPase, or 1 mM ATP for EDTA-ATPase, and stopped by addition of 10% trichloroacetic acid. The mixture was centrifuged at 3,000 × g for 5 min, and the supernatant analyzed for liberated inorganic phosphate (Pi) by the method of Martin and Doty.[11] Protein concentration was determined by the biuret method.[12]

Results and Discussion

Effect of EDC on the Thermal Gelation

The effect of EDC on the thermal jelly-strength of oval filefish meat paste is shown in Fig. 1. It was indicated from these curves that the addition of EDC to the muscle paste markedly enhanced the jelly-strength values all over the temperature tested. The jelly-strength often went over 1,000 g·cm for thermal gel of the meat paste to which was added 0.5% EDC. It is noteworthy that the gel-disintegration at 60°C was not observed on 0.5% EDC-meat pastes. Fig. 2 shows the supperssing effect by EDC on the gel-disintegration of the meat paste. The suppressing effect increased markedly by the concentration more than 0.1% EDC. The addition of 0.2% EDC showed 90% suppression effect. It seemed likely that some protein complex in the paste was formed by the cross-linking with EDC.

As is well known, oval filefish myosin paste results in remarkable gel-disintegration during heating at 60°C.[5,13] Fig. 3 presents the effect of EDC on the gel-disintegration of oval filefish myosin. The EDC treatment at 20°C for 60 min thoroughly inhibited the gel-disintegration of myosin as well as meat paste. The EDC-treatment for 5 min, however, seemed to be insufficient for the cross-linking of with EDC, since the gel-

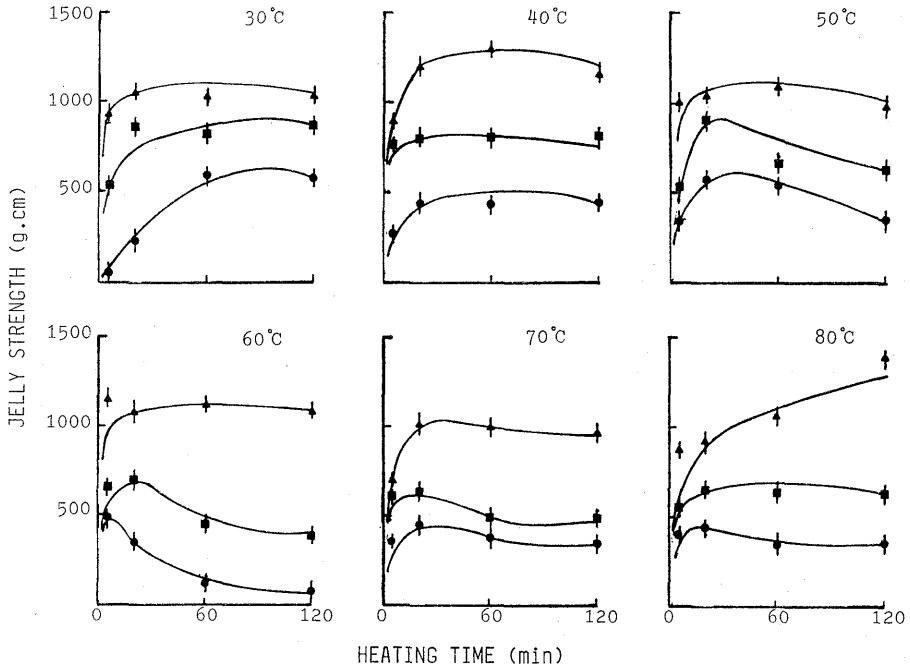

Fig. 1. Effect of EDC on the thermal jelly-strength of oval filefish meat paste. The vertical bars indicate the standard deviations from 5 experiments.
●: Without EDC, ■: With 0.2% EDC, ▲: With 0.5% EDC.

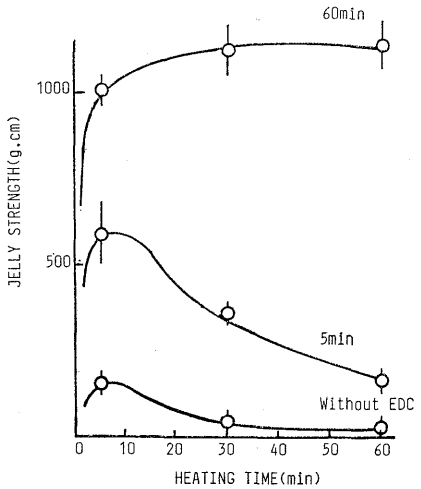

Fig. 2. Suppression by EDC for the gel disintegration of oval filefish meat paste. The value of disintegration index was expressed at the ratio of [1-(jelly-strength at 60°C for 120 min/jelly-strength at 50°C fro 20 min)]. The bars indicate the standard deviations from 5 experiments.

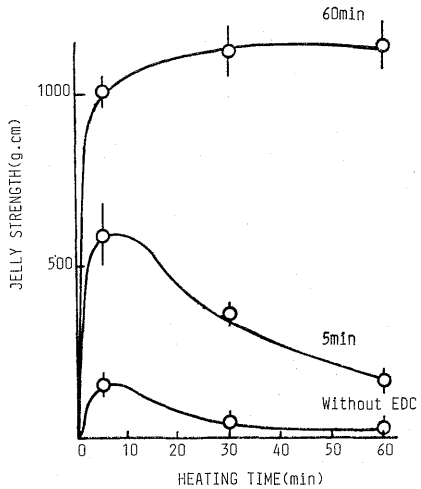

Fig. 3. Effect of EDC on the disintegration of oval filefish myosin gels at 60°C. To myosin paste was added 0.2% EDC. After incubation at 25°C for 5 or 60 min, EDC-myosins were heated at 60°C. The bars indicate the standard deviations from 5 experiments.

disintegration at 60°C occurred to a certain degree. It has been reported that the cross-linking reaction catalyzed with EDC forms covalent bonds between amino and carboxyl groups in the contact area of two proteins (actin-myosin head interaction region).[14,15] Therefore, in our experiment, it will

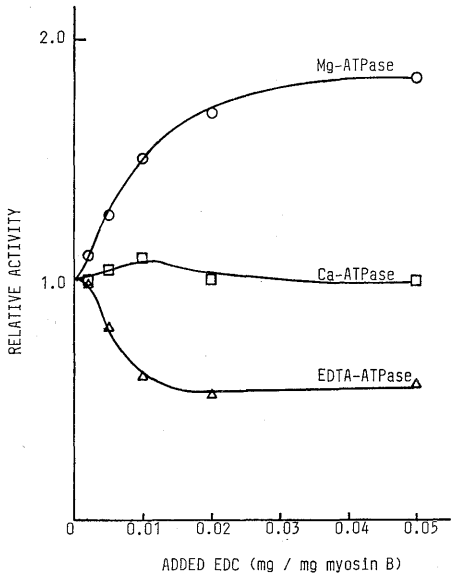

Fig. 4. Changes in the activities of oval filefish actomyosin ATPases due to EDC treatment.

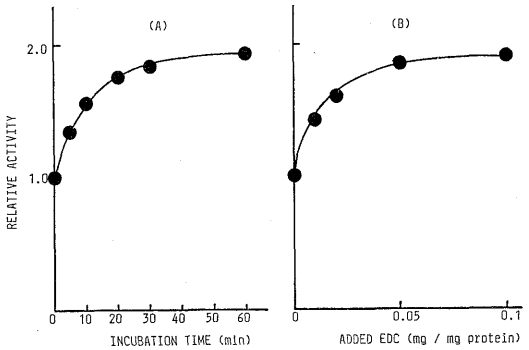

Fig. 5. Activation of acto-S-1 Mg-ATPase due to EDC-treatment.
A: Time course of acto-S-1 Mg-ATPase by adding EDC (0.05 mg/mg protein)
B: Mg-ATPase activities after the cross-linking reaction at 20°C for 60 min with varying amounts of EDC.

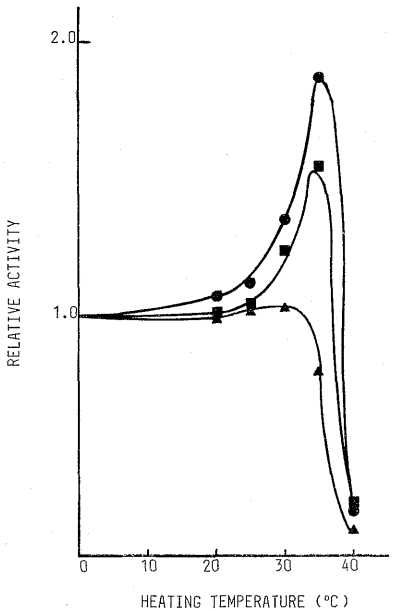

Fig. 6. Effect of EDC-treatment on the thermal activation of actomyosin Mg-ATPase.
The actomyosin ATPase without EDC (●), with 0.005 mg EDC (■), with 0.05 mg EDC (▲)/mg protein were heated at 25°, 30°, 35°C, and 40°C for 5 min, respectively.

be necessary to identify the structural features of covalently cross-linked myosin complexes with EDC. From the facts described above, the question is whether the cross-linked area by EDC is between myosin head portions, between tail portions, or between both portions.

Effect of EDC on the Actomyosin ATPases

The changes in the activities of actomyosin ATPases due to EDC-treatment are given in Fig. 4. Though the actomyosin Ca-ATPase remained almost unchanged regardless of the addition of EDC, the Mg-ATPase activity greatly increased, and the EDTA-ATPase decreased markedly. From the fact that the stimulation of Mg-ATPase and a decrease of EDTA-ATPase occurred simultaneously, it seemed that there is a possibility of the formation of cross-linking between myosin head and actin by EDC.

This possibility was also confirmed by the experimental results obtained by acto-S-1 Mg-ATPase. Fig. 5 shows the changes in acto-S-1 Mg-ATPase activity due to EDC-treatment. The result (A) provides the rate of EDC-induced cross-linking reaction of acto-S-1. The activation by cross-linking at 20°C approached a plateau after 60 min. As is evident from the figure (B), the activity was stimulated with the increase of EDC concentration (0.01 mg~0.1 mg EDC/mg protein). On the other hand, the decrease of acto-S-1 EDTA-ATPase was taken place (data not shown). From the results obtained by Figs. 4 and 5, it was found that the interaction between myosin and actin became very strong, being affected by the EDC treatment.

The effect of EDC-treatment on the thermal activation of actomyosin Mg-ATPase is presented

in Fig. 6. It has been reported that the thermal activation of Mg-ATPase is caused by some change of the interaction between myosin and actin.[16] As shown in Fig. 6, the EDC-treatment depressed the thermal activation. This will be due mainly to the strong interaction resulting from the covalently cross-linked myosin and actin complex with EDC. It appeared that the activation during heating near 35°C was unchanged as a result of the strong interaction. Further study on the thermal reaction of myosin and actin by employing EDC-treatment will be needed.

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