Bangladesh J. Fish. Res., 1(1) : 73-82
Bangladesh Fisheries Research Institute

The 66 k-Da protein identified as a light meromyosin is involved in the setting of surimi

A.K.M. Nowsad Alam[1,*], and E. Niwa

Faculty of Bioresources, Mie University, Kamihama 1515, Tsu, Mie 514, Japan
[1] Department of Fisheries Technology, Bangladesh Agricultural University Mymensingh 2202, Bangladesh

** Corresponding author*

Abstract

The 66 kilo-Dalton (kDa) protein split off from the crosslinked myosin heavy chain (CMHC) formed due to the setting of Alaska pollack surimi, frozen-storage of Pacific cod flesh, and vinegar-curing of Pacific mackerel mince was identified as a light meromyosin (LMM). Puncture and stress-relaxation tests showed that the actomyosin subunits (AMS) of Alaska pollack surimi, upon setting at 30°C, transformed into gel, although the elasticity of this gel was very low when compared to the gels from surimi or actomyosin (AM). Electrophoretic studies showed that the band due to LMM in the gel from AMS gradually disappeared with the progress of setting but higher molecular weight polymer did not form. The intensity of the bands due to other myosin sub-fragments decreased a little. The findings suggest that at setting temperature, LMM of MHC molecule leads to an unfolding resulting in an intramolecular aggregation through non-covalent interactions, and thus plays a significant role in the crosslinking of MHC.

Key words : Surimi, Light meromyosin, Non-covalent aggregation

Introduction

In the manufacturing process of kamaboko, a viscous water-washed and salt-ground fish paste (surimi paste) is usually heated at a low temperature (0-40°C) prior to cooking at a higher temperature (80-90°C). This process of heating at low temperature, called "setting", is performed to improve the elasticity of kamaboko. The surimi paste turns to an elastic, semi-transparent gel, called set gel or "suwari" in Japanese, at this time. During setting myosin heavy chain (MHC) of fish myosin crosslinks to form high molecular weight crosslinked myosin heavy chain (CMHC) (Lee *et al.*, 1990, Numakura *et al.* 1985, Numakura *et al.*1990).

In our previous experiments (Nowsad *et al.* 1993a, Nowsad *et al.* 1993b), it was observed that the CMHC was formed during the setting of Alaska pollack surimi by the aggregation of MHC and 66 kilo-Dalton (kDa) protein through weak hydrophobic and hydrogen bonds, from the fact that the CMHC split into MHC

and 66 kDa protein upon repeated extraction with concentrated urea and electrophoresis. Some low molecular weight protein components including 66 kDa protein were also split off from the CMHC formed during frozen-storage of Pacific cod flesh and dehydration of Alaska pollack surimi (Niwa *et al.* 1993). The results suggested that the 66 kDa protein might play an important role in the crosslinking of MHC. In this study, the 66 kDa protein was tentatively identified as a light meromyosin (LMM) and the role of this MHC subfragment in the setting of surimi was examined.

Materials and methods

SA-grade (Super-A grade) unsalted frozen surimi of Alaska pollack from Golden Alaska Seafoods Inc., Seattle, WA, U.S.A. was used. Pacific cod Gadus macrocephalus and Pacific mackerel *Pneumatophorus japonicus* were purchased from a retail fish store in Tsu city, Japan. Both trypsin (bovine pancreas) and trypsin inhibitor (soybean) were obtained from Sigma Chem. Co. U.S.A.

Extraction of AM

Mince of Alaska pollack surimi and dorsal and lateral muscles of Pacific cod were washed three times with 5 volumes of 3.38 mM NaH_2PO_4-15.5 mM Na_2HPO_4 (pH 7.5), followed by centrifugation at 6,000 x g for 10 min. AM was extracted for 3 h with 5 volumes of 0.8 M KCl-3.38 mM KH_2PO_4-20.5 mM Na_2HPO_4 (pH 8.0), followed by a dilution precipitation with water. One part of AM was concentrated by dialyzing against polyethylene glycol # 20,000 and the other part was dissolved in 0.5 M KCl-0.05 M Tris-maleate buffer (pH 6.2) for subsequent digestion by trypsin. All the procedures were done at 4°C.

Preparation of AM subunits containing 66 kDa protein {AM(S) }

AM was digested with trypsin at an enzyme myosin ratio of 1 : 100 (w/w) at 10°C for 2 h at an AM concentration of 15 mg /ml in 0.5 M KCl-0.05 M Tris-maleate buffer (pH 6.2) according to the method of Tsuchiya and Matsumoto (1975). The reaction was terminated by the addition of a 3 fold higher by weight of soybean trypsin inhibitor solution. The digest was dialyzed against distilled water overnight, centrifuged at 30,000 x g for 30 min and dehydrated by dialyzing against polyethylene glycol # 20,000. All the products were used within a week.

Setting, frozen-storage, and vinegar-curing

Thawed surimi, AM, and AM(S) from Alaska pollack were, respectively, ground together with 3 % NaCl, 1 % sterilizer (Ueno Fine Chem. Co., Japan, Solmighty), and varied amount of water to maintain a final moisture content of 88

% by a mortar for 10 min at 4°C. The resulting pastes were stuffed into glass tubes (10 mm in inner diameter, 45 mm in length), set in a water bath at 30°C after wrapping both ends by parafilm (American National Can, Greenwich, CT. U.S.A.) and cooled in running tapwater.

For frozen-storage, 1 g of Pacific cod mince kept in a stoppered Erlenmeyer flask was stored in a freezer at -20°C for 30 days and thawed at room temperature before the preparation of sample for electrophoresis. For vinegar-curing, 1 g of Pacific mackerel paste ground with 3 % NaCl was stuffed into cellophane tube and cured in 5 % acetic acid at 4°C for 48 h.

Measurement of elasticity

a. Puncture test

Puncture test of the set gels sliced into 15 mm height was done by using a rheometer (Fudoh Kogyo Co., Japan, NRM 2010J-CW) with a spherical plunger (5 mm in diameter) at a table speed of 6 cm/min.

b. Stress-relaxation test

The same rheometer with a flat plunger (20 mm in diameter) was used to carry out the compression stress-relaxation test. The gel of the same shape was compressed at a table speed of 30 cm/min with a constant strain of 0.1. Stress-relaxation curves recorded for five min after compression were analysed by a four-element mechanical model where two sets of Maxwell's model were connected each other in parallel. An instantaneous elastic modulus of this mechanical model (G0), the elastic modulus of a spring coil of the Maxwell's model showing the longer relaxation time (G1), the modulus of another Maxwell's model showing the shorter relaxation time (G2), the viscosity of a dashpot constructing the former model (h1), and the viscosity of a dashpot of the latter model (h2) were obtained by the progressive approximate method (Tobolsky and Murakami 1959) through the following equation:

$$P(t) = e0 \left(\sum_{i=1}^{n} Gi\ e^{-t/ti}\right)$$

where, $P(t)$ = stress, e_o= constant strain, t = time, G_i = elastic modulus of i-th element, ($G_o = G_1 + G_2 + ...+ G_n$, the instantaneous elastic modulus); and $T_i = \eta i / G_i$, η is the viscosity of the i-th element.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of the gels, frozen-stored flesh, vinegar-cured meat, AM and AM(S) were carried out as reported previously (Nowsad *et al.* 1993a) after dissolving in 8 M urea- 2 % SDS-2 % 2-mercaptoethanol-20 mM Tris-HCl (pH

8.0). AM and digested AM were dissolved in 0.5 M KCl-0.05 M Tris-maleate (pH 6.2), dialyzed against the same buffer and electrophoresed as above (op cit).

Extraction of CMHC

CMHC remained at the top of the disc gel after the SDS-PAGE was extracted in 8 M urea-2 % SDS-2 % 2-mercaptoethanol-20 mM Tris-HCl (pH 8.0) by the method described previously (Nowsad *et al.* 1993a).

Results and discussion

In Fig.1, SDS-PAGE patterns are shown for Alaska pollack surimi set at 30°C for 10 h (A), Pacific cod mince frozen-stored at -20°C for 30 days (B) and Pacific mackerel paste cured in acetic acid for 48 h (C). Three main bands were observed in all the discs - at their top as CMHC, at 205 kDa as MHC and just below 45 kDa as actin (Ac), similarly as in the case we described before (Nowsad *et al.* 1993a, Niwa *et al.* 1993). The intensity of MHC bands decreased with the progress of setting of Alaska pollack surimi, frozen- storage of Pacific cod mince and vinegar-curing of Pacific mackerel paste and in correspondence, the intensity of CMHC bands increased in all the cases. The bands for Ac did not change. The results supported the views that with the progress of either setting or frozen-storage or vinegar-curing MHCs were crosslinked to higher molecular weight CMHC.

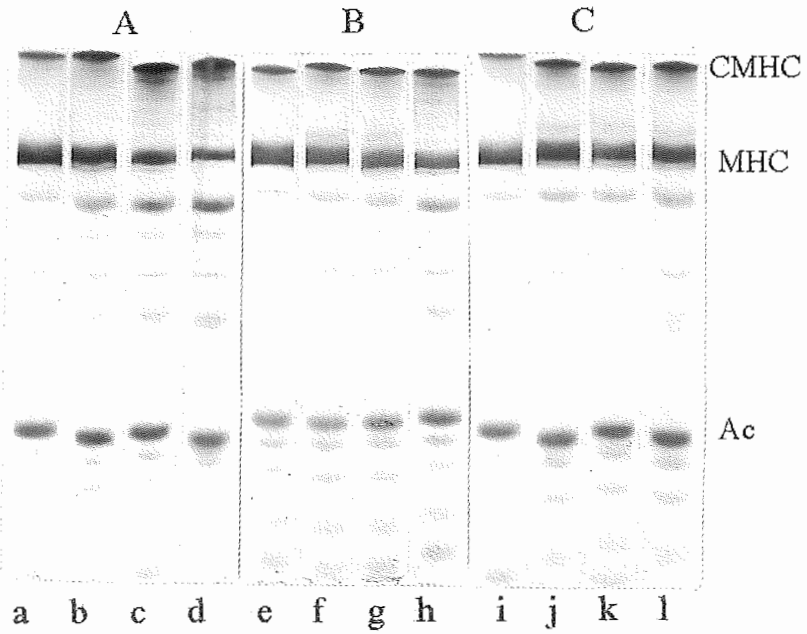

Fig.1. SDS-PAGE patterns for setting (A), frozen-storage (B) and vinegar-curing(C) of fish pastes. a, salt-ground Alaska pollack surimi; b, that set at 30°C for 1 h; c, that set for 5 h; d, that set for 10 h; e, Pacific cod mince; f, that frozen-stored at -20°C for 10 d; g, that stored for 20 d; h, that stored for 30 d; i, Pacific mackerel paste; j, that vinegar-cured for 12 h; k, that cured for 24 h; l, that cured for 48 h.

Fig. 2 shows the SDS-PAGE pattern for AM, digested AM, and CMHC extracts. CMHCs were extracted from the discs d, h and l for the setting of Alaska pollack, frozen-storage of Pacific cod and vinegar-curing of Pacific mackerel, respectively. These CMHC extracts were electrophoresed to the discs o, p and q of Fig. 2, respectively. In all of these three discs it was seen that CMHCs formed by the above treatments, were dissociated to MHC and 66 kDa protein subunit. The disc m was for AM extracted from Alaska pollack and the disc s was that from Pacific cod. The disc M was for the molecular weight marker. However, by the digestion with trypsin, MHC molecules of AMs from both Alaska pollack and Pacific cod were cleaved into 170, 145, 87, 78, and 66 kDa subfragments (disc n and r). Each mobility was in good agreement with that of the tryptically digested abalone myosin (Asakawa and Azuma 1990) in which the first (170 kDa) was assigned to an intra-cleavage of MHC, the third (87 kDa) to myosin subfragment-1 (S-1), and the last (66 kDa) to LMM. Chan *et al.* (1993) purified LMM from cod and herring myosin at a molecular weight of 66 kDa. During prolonged digestion by a-chymotrypsin and trypsin, thermally acclimated carp myosin was cleaved into various subfragments, where a number of protein bands corresponding to the molecular weight from 66 to 70 kDa were indentified to be the LMM (Watabe *et al.* 1992). From the comparison of these results, 66 kDa subfragment split off from CMHCs could also be assigned to LMM, although it was still obscure whether the LMM was formed after the aggregation of MHC or from MHC before its aggregation and then entangled with the parent MHC molecule to form CMHC.

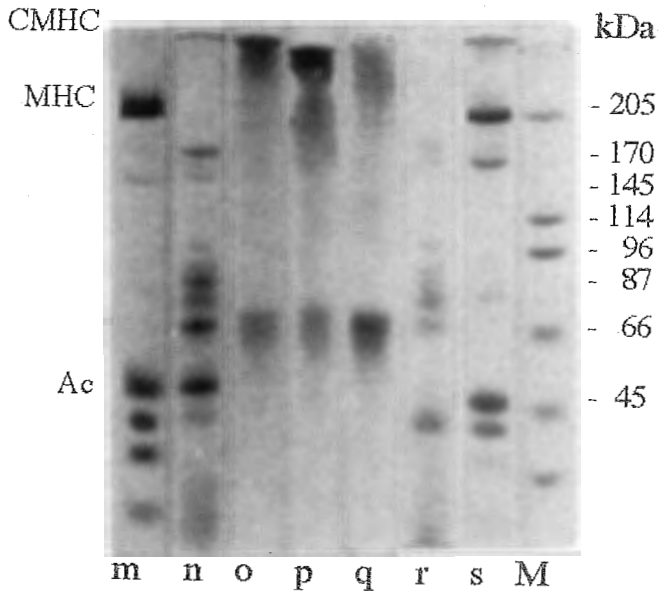

Fig. 2. SDS-PAGE patterns for AM, tryptically digested AM and CMHC extracts. m, AM of Alaska pollack; n, that digested by trypsin; o, CMHC extract from setting; p, CMHC extract from frozen-storage; q, CMHC extract from vinegar curing; r, tryptically digested AM of Pacific cod; s, AM of Pacific cod; M, molecular weight marker.

Several studies (Chan *et al.* 1993, Samejima *et al.* 1981, Sano *et al.* 1990, Taguchi *et al.* 1987) have demonstrated an active involvement of LMM in the MHC aggregation phenomenon, where most of the works have been done with purified LMM from different fish sources. The dynamic viscoelastic behaviour and turbidity studies of isolated carp heavy meromyosin (HMM) and LMM suggested that the initial gel formation was attributed mainly to LMM at 30-44°C (Sano *et al.* 1990). However, the purification process can affect the stability of protein (Otani *et al.* 1983, Park and Lanier 1989). It is important to determine the gelling characteristics of model protein not only in the purified form but also in a less purified system (Beas *et al.* 1991). Therefore, in order to understand the role of MHC subunits including LMM in surimi gelation, AM(S) from Alaska pollack was used in the present study. The objective was to understand the influence and interactions of these subunits in the crosslinking phenomenon in a condition of set gel which more or less resembled set gel of AM, where MHC was completely cleaved by a prolonged digestion with the enzyme but the subfragments were not isolated or separated.

Fig. 3 shows the elasticity of set gel in respect of puncture test. Both surimi and AM showed increased setting ability at 30°C as their breaking strength and breaking deformation increased rapidly at the initial stage and then gradually till 6 h of setting. On the other hand, the paste of AM(S) although transformed into gel, the breaking strength and breaking deformation were very low and reached a constant after an increase till 4 h. The elastic modulii and viscosities of these gels are presented in Fig. 4. G0, G1, G2, h1, and h2 rapidly increased in the gels from surimi and AM till 6 h of setting, but slowly increased in the gel from AM(S). The increment of elasticity in the gel from AM(S) was very small when compared to the strong elasticity of surimi and AM. Nevertheless, the results confirmed that the gelling characteristics of MHC also persisted in its subfragments. The influence of Ac in this gelation could be nullified, since it has been demonstrated from the turbidimetric and electrophoretic studies that actin does not involve in the gelation and protein crosslinking phenomena (Samejima *et al.* 1969).

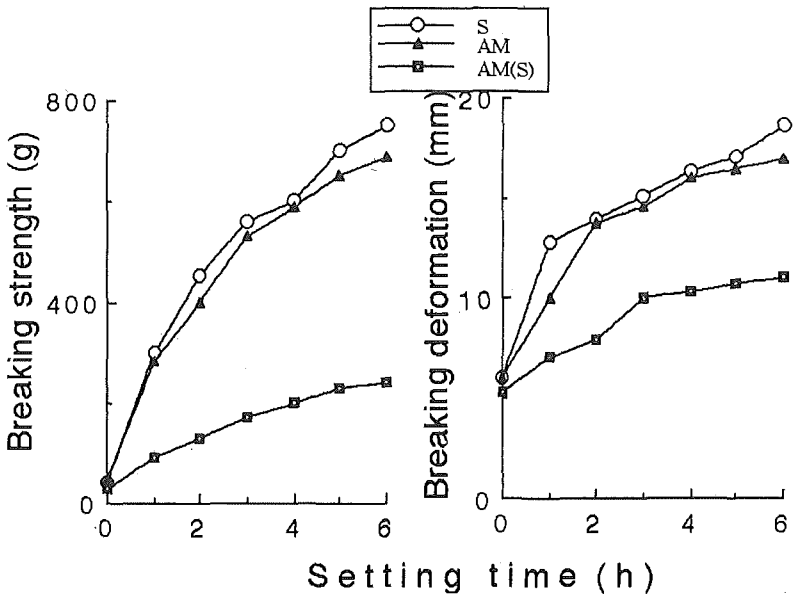

Fig. 3. Breaking strength and breaking deformation of the gel prepared from surimi (S), actomyosin (AM) and actomyosin subunits containing 66 kDa protein {AM(S)} from Alaska pollack.

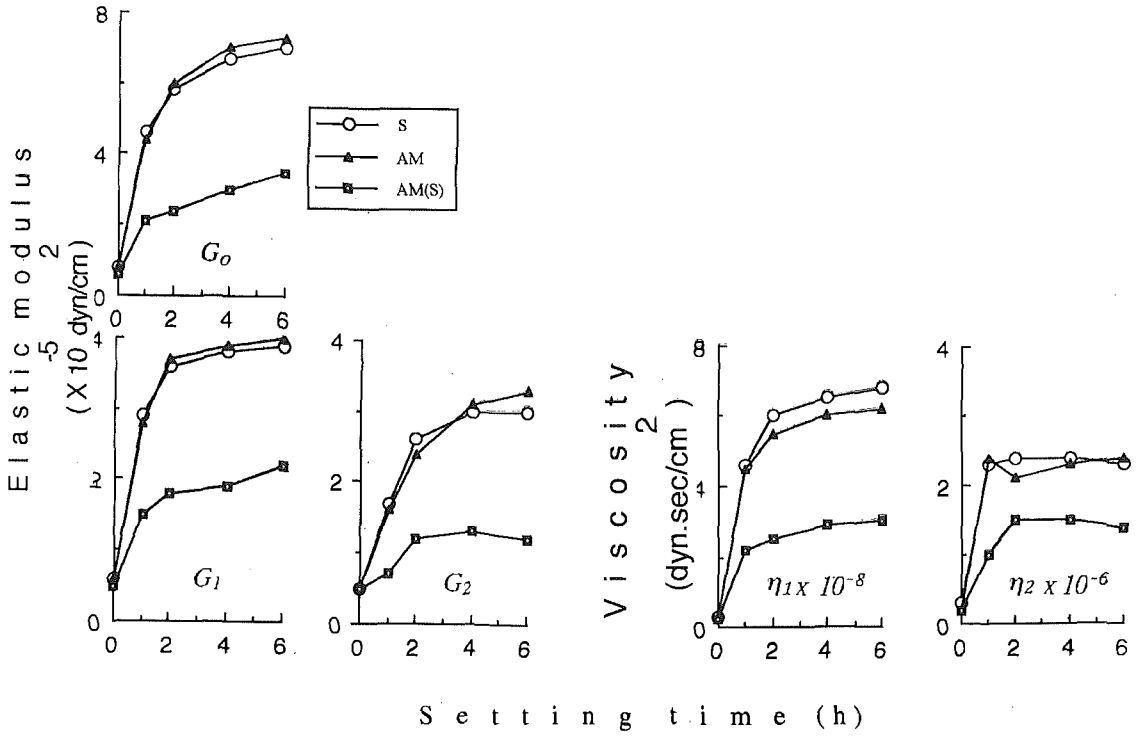

Fig. 4. Compression stress-relaxation of the gel prepared from surimi, AM and AM(S) of Alaska pollack.

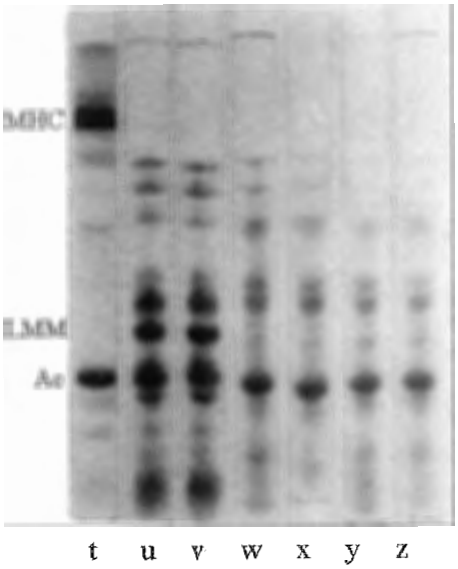

Fig. 5. SDS-PAGE patterns for the gel prepared from the AM(S) pastes. t, AM of Alaska pollack; u, AM(S); v, salt-ground AM(S); w, that set at 30°C or 1 h; x, that set for 2 h; y, that set for 4 h; z, that set for 6 h.

Fig. 5 shows the SDS-PAGE pattern for the gel prepared from the paste of AM(S). Due to prolonged digestion, MHC was completely cleaved and most of the other higher molecular weight subunits like HMM and S-1 split into smaller units, but LMM remained intact. The role of MHC subunits in the low temperature gelation had been studied by many authors (Chan *et al.* 1993, Samejima *et al.* 1981, Sano *et al.* 1990, Taguchi *et al.* 1987). However, as mentioned earlier, the objective of this study was to find whether the 66 kDa protein, tentatively identified as a LMM, could go crosslinking in a less purified form. It was observed from the electrophoretic study that during setting at 30°C the band due to LMM disappeared (discs w, x, y, z) and the intensity of the bands due to other MHC subfragments gradually decreased. However, high molecular weight polymer was not formed. Since the fact that only intermolecular interactions would lead to formation of high molecular weight polymers (Chan *et al.* 1992), the polymerization occurred among MHC subfragments in this study as observed due to their disappearance during setting would be intramolecular. Furthermore, the polymerization of subunit proteins during setting was predominantly non-covalent, since the enzyme transglutaminase, responsible for the occurrence of covalent crosslinking, might be washed away during AM extraction and digestion process, as had been explained previously (Nowsad *et al.* 1994). The demonstration of non-covalent crosslinking by simple SDS-PAGE system is very often precluded because SDS readily breaks hydrogen, hydrophobic and electrostatic bonds (Chan *et al.* 1992). However, from the results that the intensity of the band due to LMM during setting decreased more among the subfragments in the present experimental condition, it was presumed that LMM involved more in the crosslinking process at the temperature around 30°C. Samejima *et al.* (1981) mentioned that the tail

portion of myosin rod in rabbit myosin was apparently responsible for the formation of a gel network, presumably by hydrophobic interaction. The same authors also reported such entanglement of protein molecules at temperatures around 30-35°C in the setting of fish paste. Gill and Conway (1989) studied chymotryptic cleavage of thermally aggregated cod myosins and concluded that the initial stages of thermal crosslinking of myosin were mediated primarily by the HMM-S-2 and LMM region of heavy chain, i.e., by the myosin tail rather than the head. Chan *et al.* (1992) reported the involvement of both HMM and LMM in the thermal crosslinking of cod and herring myosin and explained a mechanism which expressed the view that an initial aggregation might be occurred by the unfolding and interaction at 30-40°C range and further aggregation might be mediated by the interaction of LMM to form clusters of aggregates at 40-55°C. The results of the present study apparently differ from that of Chan *et al.* (op cit) since LMM was mostly crosslinked within one hour of setting at 30°C. In fact, larger myosin subfragments like HMM and S-1 were mostly cleaved into smaller ones in this study. Therefore, the exact influence of these subfragments can not be understood and explained from these results. Nonetheless, it seems that MHC molecule may lead to an unfolding of the super helix of the tail at setting temperatures, exposing hydrophobic residues to the polar environments, resulting in the intramolecular crosslinking through non-covalent bonds among the subfragment proteins that cleaved from the tail portion of the molecule. This may be further entangled with the intermolecular crosslinking of MHC itself and locked up in CMHC at the top of the SDS-PAGE disc gel which could split off again from CMHC in concentrated urea, as we explained elsewhere (Nowsad *et al.* 1993).

References

Asakawa, T. and N. Azuma, 1990. Proteolytic digestion of myosin from abalone Haliotis discus smooth muscle. *Nippon Suisan Gakkaishi*, **56:** 297-305.

Beas, V.E., J. R. Wagner, M. C. Anon and M. Crupkin, 1991. Thermal denaturation in fish muscle proteins during gelling: effect of spawning condition. *J. Food Sci.*, **56:** 281-284.

Chan, J. K., T. A. Gill and A. T. Paulson 1992. Cross-linking of myosin heavy chains from cod, herring and silver hake during thermal setting. *J. Food Sci.*, **57:** 906 - 912.

Chan, J. K., T. A. Gill and A. T. Paulson, 1993. Thermal aggregation of myosin subfragments from cod and herring. *J. Food Sci.*, **58:** 1057-1069.

Gill, T. A. and J. T. Conway, 1989. Thermal aggregation of cod *(Gadus morhua)* muscle proteins using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as a zerolength cross-linker. *Agric. Biol. Chem.*, **53:** 2553-2562.

Lee, N., N. Seki, N. Kato, N. Nakagawa, S. Terui and K. Arai, 1990. Gel forming ability of myosin heavy chain in salted meat paste from threadfin bream. *Nippon Suisan Gakkaishi*, **56:** 329-336.

Niwa, E., S. Suzuki, A.K.M.A. Nowsad and S. Kanoh, 1993. Lower molecularfish proteins separated from the band around that of cross-linked myosin heavy chain. *Nippon Suisan Gakkaishi,* **59:** 1013-1016.

Nowsad, A.K.M.A., S. Kanoh and E. Niwa, 1993a. Electrophoretic behavior of crosslinked myosin heavy chain in suwari gel. *Nippon Suisan Gakkaishi,* **59,** 667-671.

Nowsad, AKM.A., S. Kanoh and E. Niwa, 1993b. Aggregation of myosin heavy chain while grinding surimi and setting its paste. *Nippon Suisan Gakkaishi,* **59:** 1957.

Nowsad, AKM. A., S. Kanoh and E. Niwa, 1994. Setting of transglutaminase-free actomyosin paste prepared from Alaska pollack surimi. *Fisheries Science,* **60:** 295-297.

Numakura, T., N. Seki, I. Kimura, K. Toyoda, T. Fujita, K. Takama and K. Arai, 1985. Cross-linking reaction of myosin in the fish paste during setting (SUWARI). *Nippon Suisan Gakkaishi,* **51:** 1559-1565.

Numakura, T., I. Kimura, K. Toyoda and T. Fujita, 1990. Temperature-dependent changes in gel strength and myosin heavy chain of salt-ground meat from walleye pollack during setting. *Nippon Suisan Gakkaishi,* **56:** 2035-2043.

Otani, O., S. Hikichi, K. Nishita, T. Sekii and K. Arai, 1983. A loss of actin from shellfish myofibrils and fish myosin B during wash-treatment. *Nippon Suisan Gakkaishi,* **49:** 415-424.

Park, J. W. and T. C. Lanier, 1989. Scanning calorimetric behavior of tilapia myosin and actin due to processing of muscle and protein purification. *J. Food Sci.,* **54:** 49-51.

Samejima, K., Y. Hashimoto, T. Yasui and T. Fukazawa, 1969. Heat gelling properties of myosin, actin, actomyosin and myosin-subunits in a saline model system. *J. Food Sci.,* **34:** 242-245.

Samejima, K., M. Ishioroshi and T. Yasui, 1981. Relative roles of the head and tail portions of the molecule in the heat-induced gelation of myosin. *J. Food Sci.,* **46:** 1412-1418.

Sano, T., S. F. Noguchi, J. J. Matsumoto and T. Tsuchiya, 1990. Thermal gelation characteristics of myosin subfragments. *J. Food Sci.,* **55:** 55-58.

Taguchi, T., M. Ishizaka, M. Tanaka, Y. Nagashima and K. Amano, 1987. Protein-protein interaction of fish myosin fragments. *J. Food Sci.,* **52 :** 1103-1104.

Tobolsky, A.V. and K. Murakami, 1959. *J. Polymer Sci.,* **40** : 443-446, (cited by Iso, N., H. Mizuno, T. Saito, F. Ohzeki and C. Y. Lin, 1983. An analysis of the stress-relaxation curve obtained from a sample of raw meat sample. *Nippon Suisan Gakkaishi,* **49:** 949-952).

Tsuchiya, T. and J. J. Matsumoto, 1975. Isolation, purification and structure of carp myosin, HMM and LMM. *Nippon Suisan Gakkaishi,* **41:** 1319-1326.

Watabe, S., G. C. Hwang, M. Nakaya, X. F. Guo and Y. Okamoto, 1992. Fast skeletal myosin isoforms in thermally acclimated carp. *J. Biochem.,* **3:** 113-122.

(Manuscript received 21 July 1996)