The Combined Effect of Transglutaminase and Protease Inhibitors on the Thermal Gelation of Actomyosin Sol from Carp and Salmon Muscles

Shaowei Ni, Hisanori Nozawa, and Nobuo Seki
Laboratory of Food Biochemistry, Faculty of Fisheries, Hokkaido University, Minato, Hakodate 041-8611, Japan
(Received December 28, 1998)

In order to improve the poor gel-formability of underutilized fish species for surimi-based products, such as carp and salmon, we attempted to induce setting effect to actomyosin gel with microbiological transglutaminase (MTGase) and to inhibit the gel weakening (modori) caused by non-proteolytic and proteolytic processes. Carp and salmon actomyosin sols (90 mg protein/g in 0.5 M NaCl-5 mM CaCl₂ at pH 7.0) without MTGase exhibited similar changes in storage modulus (G') with a temporal sharp reduction (non-proteolytic modori) at 53 and 48°C for carp and salmon, respectively, though the G' magnitude of salmon sol was twice that of carp during heating from 5 to 80°C. Compared to walleye pollack surimi gel, however, both gels were extremely weak at the end of heating.

The addition of MTGase to actomyosin sol could induce setting effect and resulted in elastic and rigid gels with a great increase in G' magnitude as well as gel breaking strength. Salmon myosin heavy chain was more easily and extensively polymerized than that of carp. Gel weakening was slightly improved by the addition of protease inhibitors, E64 and aprotinin. The effects of protease inhibitors on carp actomyosin gelation were more distinctive when combined with MTGase. For salmon actomyosin gel, the protease inhibitor had less effective due to lower remaining of endogenous proteases in the actomyosin sol. Upon the setting at 40°C, the addition of 5 unit/g MTGase substantially increased in G' and extensively reduced the non-proteolytic modori. Under identical gelling conditions, salmon actomyosin sol was capable of forming more rigid and elastic gel than that of carp.

Key words: gelation, setting, modori, transglutaminase, protease inhibitor, carp, salmon

Materials and Methods

Materials
Live carp Cyprinus carpio and salmon Oncorhynchus keta were purchased from a local store. Phenylmethylsulfonyl fluoride (PMSF), monodansylcadaverine (MDC),
and aprotinin were purchased from Sigma. N\{-N-(1-3-trans)carboxyoxiran-2-carbonyl\}-L-leucylagmatine (E64) was purchased from Peptide Institute. All other chemicals were of analytical grade from Wako Pure Chemical Industries. Microbial transglutaminase (MTGase, EC 2.3.2.13) was supplied by Ajinomoto Co. Inc. The specific activity was determined to be 260 unit/mg by the method described by Kishi et al. One unit of the activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol MDC into acetylated casein for 1 min of incubation at 25°C.

**Preparation of Actomyosin Sol**

Fish samples were filleted and skinned. The dorsal muscle was minced and washed with 0.1 M NaCl-20 mM Tris-HCl (pH 7.0) three times. The washed muscle was homogenized in 5 volumes of 0.1 M NaCl-20 mM Tris-HCl (pH 7.0). After centrifuging, the precipitated myofibrils were washed 4 times with the same buffer and passed through a layer of gauze to remove connective tissues. The myofibrils were ground with NaCl to prepare actomyosin sol which contained 90 mg protein/g of sol, 0.5 M NaCl, 5 mM CaCl₂, and 20 mM Tris-HCl (pH 7.0) at a final concentration. In another set of experiment, three different protease inhibitors, 0.1 mM E64, 0.01 mM aprotinin, and 0.2 mM PMSF, were separately mixed with actomyosin sol at the grinding step. All procedures were carried out at 0-4°C. Protein concentration was determined by the biuret method.

**Gelation and Rheological Measurement**

Actomyosin sol was deaerated, packed into plastic vessels (3.7 cm diameter, 2 cm height), and then heated at 90°C for 20 min or by a two step heating method in which the sol was incubated at 30, 40, 50, and 55°C for various durations prior to the heating at 90°C for 20 min. For rupture test, the heated samples were immediately cooled in an ice bath. They were kept at room temperature for 2 h and then removed from the vessels. Breaking strength and strain were measured by using a cylindrical plunger (5 mm diameter) at a penetration speed of 0.5 mm/sec in a rheometer (Rheonier RF3305, Yamaden). For the measurement of dynamic rheological properties, deaerated actomyosin sol (1.6 ml) was put into a cell of a Rheograph Sol (Toyo Seiki Seisakusho). Two programmed heating methods were conducted: (1) one step linear heating from 5 to 80°C at a heating rate of 2°C/min. (2) a two step heating consisting of a linear heating from 5°C to a given setting temperature (30, 40, 50 and 55°C), setting for 60 min, and followed by linear heating to 80°C (2°C/min). The values of storage modulus, G', and loss modulus, G'', were recorded as described previously. The reproducibility of the data was confirmed by duplicate runs.

**SDS-PAGE**

Degradation and polymerization of myofibrillar proteins were examined by SDS-PAGE. To prevent proteolysis during sample preparation, the sample (0.4 g) was mixed with a 7.5 ml SDS-urea solution (2% SDS, 8 M urea, 2% 2-mercaptoethanol, and 50 mM Tris-HCl at pH 8.0) preheated at 90°C. After heating for 2 min, the sample was continuously stirred at room temperature for 24 h. Electrophoresis was carried out according to the procedure of Weber and Osborn. The separating disk gel contained 2% polyacrylamide and 6 M urea-0.1% SDS. In another experiments, 10% polyacrylamide slab gels were used. Protein bands were stained with 0.12% Coomassie brilliant blue R250 in 10% acetic acid and 50% methanol.

**Results and Discussion**

**Dynamic Viscoelastic Properties**

The linear heating of carp actomyosin sol resulted in a typical rheogram with G' peaks at 36 and 45°C, and 80°C at the terminal point including a temporary sharp drop at 53°C in accordance with the result of preceding paper (Fig. 1). Under the identical experimental conditions, salmon actomyosin sol showed that G' peaks slightly shifted to lower temperatures at 30°C with a shoulder at 40°C. The G' reduction was detected at 48°C. The temporary reduction in G', which represents non-proteolytic modori, has been observed at temperatures between 50 and 60°C in different fish actomyosins and surimi, chicken and rabbit actomyosins in 0.6 M NaCl, and beef myosin at low ionic strength. This modori phenomenon was attributed by Sano et al. to the dissociation of actin from myosin and possible depolymerization of the actin filaments.

During thermal gelation, carp and salmon actomyosins exhibited a similar change pattern in G', even though the magnitude for salmon was virtually twice as high as that for carp. The difference in G' magnitude between carp and salmon actomyosins seems to be originated from their inherent nature. Corresponding to the temporary reduction of G', G' started decreasing from 43°C for carp and 40°C for salmon, which was evidence of the formation of protein networks that were less viscous in nature (data not shown). The final G' values at terminal 80°C were 5 x 10³ and 9 x 10³ dyn/cm² for carp and salmon actomyosin gels, respectively. These values were low in comparison with 26 x 10³ dyn/cm² for walleye pollock surimi gel.

As reported previously, both carp and salmon actomyosin sols are devoid of setting response. We
confirmed again these results in which $G'$ of actomyosin gels after two step heating showed similar magnitude to that of gel formed by direct heating (compare Fig. 1 and Fig. 2, CO and SO). To induce setting effect to the thermal gelation, MTGase (5 unit/g) was added to the actomyosin sol which was then treated by the two step heating method including setting at 30°C for 1 h. During the setting, $G'$ of both actomyosin sols significantly increased. However, $G'$ reduction around 50°C was not affected by the added MTGase. Subsequent heating to 80°C increased $G'$ to a great extent, demonstrating the marked effect of MTGase-induced setting. The magnitude of $G'$ at the end of heating was greater in salmon gel than in carp one. Changes in $G''$ of carp actomyosin sol was not affected by the addition of MTGase, while $G''$ of salmon sol increased with MTGase during setting and following linear heating (Fig. 2).

The reactivity of endogenous transglutaminase to salmon actomyosin sol is higher than that to carp actomyosin.9) MTGase at an identical activity level polymerized salmon myosin heavy chain more easily and extensively than did carp one. In a typical example (Fig. 3), salmon myosin heavy chain was polymerized to huge polymers which could not penetrate into a polyacrylamide gel matrix after 2 h setting at 30°C with 1 unit/g MTGase, while carp myosin heavy chain was slightly polymerized but did not produce the huge polymers. These different reactivities seem to influence $G'$ value of the final gels, thereby affecting the viscoelastic attributes of both gels.

**Effect of Protease Inhibitors**

Since muscle tissue contains many endogenous proteases, it is possible that proteases which have not been completely removed by washing can cause proteolytic modori. To determine the possible involvement of endogenous proteases in the gelation of actomyosin sol, three protease inhibitors were separately incorporated into the sol. The actomyosin sol was incubated at 30, 40, 50, and 55°C for various durations (Fig. 4). At 30°C incubation, carp actomyosin was not significantly degraded for up to 4 h, regardless of the presence and absence of protease inhibitors. At 40°C, however, myosin heavy chain was preferentially hydrolyzed and almost disappeared after 2 h incubation. A major fragment with 150 kDa was formed. The degradation of myosin heavy chain was considerably inhibited with E64, a specific cysteine protease inhibitor. Aprotinin and PMSF, serine protease inhibitors, showed no inhibitory effect on the hydrolysis of myosin heavy chain at 40°C. The severe degradation of myosin heavy chain occurred during the incubation at 50 and 55°C. The degradation was inhibited in some degree with the addition of aprotinin, while E64 and PMSF showed no effect. The results at 50°C are not shown because of the similarities to those at 55°C. The effectiveness of different protease inhibitors at different temperatures demonstrated that carp actomyosin sol was contaminated with various types of endogenous proteases having different optimal temperatures. Cathepsin L, myofibril-bound serine protease, and heat-stable alkaline protease13,25) seem to be involved in the myosin heavy chain degradation during the incubation at 40 and 50-55°C, respectively. The former two proteases have been recently isolated and characterized from carp muscle by Ogata et al.26) and Osatomi et al.27)

Salmon myosin heavy chain in the actomyosin sol was hydrolyzed during the incubation at 30°C (Fig. 5). The degradation was completely inhibited with 0.1 mM E64, suggesting the presence of cathepsin L in the salmon actomyosin. It has been well established by Yamashita and Konagaya8,28) that salmon muscle contains the high activity of cathepsin L which hydrolyzes its myofibrillar proteins, especially myosin heavy chain, during storage and proc-
Effect of TGase and Protease Inhibitors on Gelation

Fig. 4. Changes in SDS-PAGE patterns of carp actomyosin sol with or without protease inhibitors during incubation.
Carp actomyosin sols were incubated at 30, 40, and 55°C with or without 0.1 mM E64, 0.01 mM aprotinin or 0.2 mM PMSF. HC, Myosin heavy chain; Ac, actin.

Fig. 5. Changes in SDS-PAGE patterns of salmon actomyosin sol with or without E64 during incubation.
Salmon actomyosin sols were incubated with or without 0.1 mM E64 at 30°C (upper gels) and 55°C (lower gels).

Thermal Gelation with Protease Inhibitors
In order to estimate the effect of protease inhibitors on the thermal gelation of carp actomyosin sol, dynamic rheological behavior was measured during programmed heating (Fig. 6: 0, E, and A). Without the protease inhibitor, carp actomyosin sol showed a typical change in $G'$. The addition of E64 slightly increased $G'$ value during setting at 30 and 40°C. On the other hand, aprotinin was ineffective at 30 and 40°C. It slightly increased $G'$ value during 55°C setting and the final $G'$ value at 80°C was slightly higher than that without aprotinin. Therefore, the increased $G'$ value obtained by these protease inhibitors reflects inhibi-
Fig. 6. Effects of the combination of MTGase and protease inhibitors on the thermal gelation of carp actomyosin sols.
The followings were added to carp actomyosin sols: O, no additive; E, 0.1 mM E64; A, 0.01 mM aprotinin; S, 5 unit/g MTGase; SE, 5 unit/g MTGase and 0.1 mM E64; SA, 5 unit/g MTGase and 0.01 mM aprotinin. The actomyosin sols were subjected to programmed heating.

Fig. 7. Effects of the combination of MTGase and protease inhibitors on the thermal gelation of salmon actomyosin sols.
The experimental conditions and abbreviations were the same as in Fig. 6 except that salmon actomyosin was used.

ry effects on the hydrolysis of myosin heavy chain.
When the gelation of salmon actomyosin sol was carried out by the programmed heating, the addition of protease inhibitors showed no effects on G' change probably because of low contamination of proteases (data not shown because of similar results to O in Fig. 7).

Combined Effect of Transglutaminase and Protease Inhibitor
MTGase induces setting effect to thermal gelation of carp actomyosin sol in a dose dependent manner as reported in the preceding paper.13 The addition of MTGase (5 unit/g of sol) to carp and salmon actomyosin sols greatly increased G' value during setting (at 30, 40, and 55°C) and the following heating to 80°C (Figs. 6 and 7). At the terminal 80°C, the extent of an increase in G' was greater in 40°C setting than in 30°C setting for carp. Although the setting at 55°C also increased G', the increase was leveled off during the following heating. We found that non-proteolytic modori, which was detected by the temporary reduction in G' around 53°C for carp and 48°C for salmon, decreased markedly by the addition of 5 unit MTGase during the setting at 40°C. The setting temperature and MTGase level seemed to be critical for overcoming the non-proteolytic modori, because the setting at 30 and 55°C generated the non-proteolytic modori even in the presence of both MTGase and protease inhibitors. This finding may give a new insight into producing more elastic and rigid gel matrix in the thermal gelation of carp muscle protein. Overall shape of G' change during carp actomyosin gelation with MTGase at 40°C was very similar to that of walleye pollack surimi, but the magnitude was low.24
Since the setting with 5 unit MTGase or with the addition of protease inhibitors greatly improved the texture of carp actomyosin gel, the combined effect of MTGase and protease inhibitors on the thermal gelation was investigated (Fig. 6; S, SA, and SE). When carp actomyosin gelation
with the combination of MTGase and either E64 or aprotinin was carried out by the programmed heating including 30°C setting. G' value at the terminal 80°C greatly increased in aprotinin added-gel, while E64 showed no effect (compare 5E and 5A with 5 at 30°C). However, E64 effectively increased the G' value on the setting at 40°C. Aprotinin with MTGase was more effective on the gelation of actomyosin sol during heating to 80°C after 40°C setting. Aprotinin was also effective on the gelation of actomyosin sol during the setting at 55°C. As shown in Fig. 4, the addition of E64 at 40°C inhibited the hydrolysis of myosin heavy chain and resulted in the increase in G' value during the heating to 80°C. On the other hand, aprotinin considerably inhibited proteolysis at 55°C. Therefore, the inhibition of proteolysis during the heating above 50°C is more important to the formation of actomyosin gel with greater G' magnitude. The temperature range is known as modori inducing temperature.

The combined effect of MTGase and protease inhibitor on the thermal gelation of salmon actomyosin sol was also investigated (Fig. 7) and compared with that of carp as described above. The addition of MTGase greatly increased G' value during setting and the following heating to 80°C, showing the setting response to the gelation of salmon actomyosin sol. E64 or aprotinin together with MTGase slightly increased the G' value during the setting at 30, 40, and 55°C and the following heating to 80°C. The weak effect of protease inhibitors on the gelation of salmon actomyosin sol resulted from the low level of remaining proteases (Fig. 5).

To investigate the combined effect of added MTGase and protease inhibitor on final gel strength by rupture test, carp actomyosin gels containing 0.1 mM E64 and MTGase at various activity levels (1, 3, and 5 unit/g of gel) were prepared by setting at 30°C for up to 4 h and 40°C for up to 2 h, followed by final cooking at 90°C for 20 min (Fig. 8). The higher gel strength resulted from the samples for longer setting time at 30°C and at higher MTGase activity level. The setting effect induced by MTGase was significant. In contrast, the effect of added E64 on the final gel strength was only distinct in lower MTGase levels, suggesting that proteolytic gel weakening was compensated by the cross-links formed with MTGase. Furthermore, the heating at 90°C after setting inhibited the proteolysis due to high heating rate at 5.56°C/min as compared with 2°C/min in dynamic rheological tests. The quick heating above 4°C/min has appreciable effect to reduce the degradation of myosin heavy chain. When actomyosin sol with 3 or 5 unit/g MTGase with or without E64 was set at 40°C for more than 1 h, the texture of final gels was changed from elastic to hard and crumbly. The change resulted in weakening of gel strength; i.e. the force required to rupture the gel decreased markedly due to a crack occurring during the penetration of plunger and the breaking strain also decreased. The gel strength data of hard and crumbly gels did not necessarily agree with the strong G' magnitude of the final gels in dynamic rheological measurements. Therefore, the observed gel weakening, which should not be interpreted as modori, seemed to be generated from the overproduction of cross-links by MTGase reaction as reported previously, in accordance with the results obtained by Seguro et al. and, in addition, from the limita-

Fig. 8. Effects of various combinations of MTGase and E64 on the gel strength of carp and salmon actomyosin gels.

Actomyosin sols with the combination of MTGase and 0.1 mM E64 were set at 30 and 40°C prior to heating at 90°C for 20 min. , with MTGase; , with MTGase and E64.
tion of the method of rupture test.

Salmon actomyosin gels showed higher breaking strength than that for carp under the same heating conditions with the same levels of MTGase and E64. Saeki et al.\(^\text{30}\) reported that the gelation of salmon surimi required protease inhibitors for the production of strong elastic gel matrix due to remaining proteases at higher levels. In salmon actomyosin sol, however, the remaining protease activity was low, suggesting that the myofibrill-bound proteases were low in the muscle and a major contaminating protease, cathepsin L, had been removed by the repeated washings.

In conclusion, MTGase-mediated setting, which is induced by the formation of myosin heavy chain cross-links, improves the thermal gel-forming properties of carp and salmon actomyosin sols. Furthermore, it overcomes the temporary \(G'\) reduction (non-proteolytic \textit{modori}) at 40°C. Proteolytic \textit{modori} was inhibited by the addition of E64 and aprotinin. The combined effect of MTGase and protease inhibitors on the gel properties is more distinct in carp actomyosin gels than in those of salmon.

References


21) A. Egelandsdal, K. Fretheim, and K. Samejima: Dynamic rheological behaviour of salmon actomyosin sol, however, the remaining protease activity was low, suggesting that the myofibrill-bound proteases were low in the muscle and a major contaminating protease, cathepsin L, had been removed by the repeated washings.


