Myosin Denaturation and Cross-linking in Alaska Pollack Salted Surimi during Its Preheating Process as Affected by Temperature

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Myosin denaturation and cross-linking during the preheating process of Alaska pollack salted surimi at various preheating temperatures were studied. Thermal gel properties of preheated-gel and those after heating at 90°C were measured. At 15°C and 25°C, loss of salt- and urea-solubility of myosin preceded ATPase inactivation and cross-linking. At 35°C, a very quick ATPase inactivation and loss of salt-solubility was followed by a loss of urea-solubility of myosin. Myosin cross-linking reaction followed these changes. Preheating at these temperatures increased the breaking force of the two-step heated gel. At 45°C, despite a quick loss of ATPase and salt-solubility, urea-solubility remained high and no cross-linking was observed. Furthermore, there was no increment in breaking force upon preheating at this temperature. Thus, myosin aggregates, as revealed by the loss of urea-solubility as well as the cross-linking reaction, were important in improving thermal gel properties.

Keywords: surimi, myosin denaturation, Alaska pollack, cross-linking, aggregation, setting

Introduction

Frozen surimi of Alaska pollack is a material distributed worldwide for use in thermal gel products. Myosin plays a vital role in the thermal gel formation of meat, including surimi (Arai *et al.*, 1970; Samejima, 1981). Shimizu *et al.* (1981) extensively studied the thermal gel forming ability of muscle protein in many fish species, and concluded that it is strongly dependent on the species. Salting is the essential step in thermal gel formation, dissolving myofibrillar proteins before heating. To improve the gel strength of the final thermal gel, Japanese manufacturers often employ a preheating process before cooking at a high temperature (Kato *et al.*, 1984); this process is also termed as ‘setting’. A well-known event occurring in the myosin molecule during the preheating process of salted surimi is the progressive production of cross-linked myosin (Numakura *et al.*, 1985; Kimura *et al.*, 1991). This reaction is thought to be catalyzed by endogenous transglutaminase (EC 2.3.2.13, TGase) (Seki *et al.*, 1990; Araki and Seki, 1993; Park *et al.*, 2003).

It is also known that the preheating of salted surimi causes myosin denaturation. The myosin of Alaska pol-

lack is very unstable, as assessed by thermal inactivation of Ca^{2+} -ATPase (Hashimoto *et al.*, 1982), and the presence of sorbitol in surimi prevents its denaturation during storage. The optimal temperature for preheating differs from species to species and is strongly correlated with the thermal stability of myosin (Hashimoto *et al.*, 1985). We have established techniques to study myosin denaturation in preheated salted surimi (Konno and Imamura, 2000). We found that myosin in Alaska pollack surimi showed a rapid loss of solubility in 0.5 M and 8 M urea, which was a faster event than Ca^{2+} -ATPase inactivation when studied at 25°C. For practical reasons, the incubation temperature can be modified by the producer. For example, raising the temperature is the simplest way to shorten the process. However, incubation at high temperatures sometimes results in serious quality problems in the final thermal gel.

There is little information on the correlation between myosin denaturation during the preheating process of salted meat paste and gel formation. In the present study, we analyzed myosin denaturation, especially myosin aggregation as assessed by loss of salt- and urea-solubility, as well as Ca^{2+} -ATPase activity, during the heating at various temperatures (15°C to 45°C). Myosin cross-linking was also studied, as this reaction plays an important role in gel formation. To ac-

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cess the consequence of preheating salted surimi, we also assessed the change in the breaking force for the preheated and final gel after heating at 90°C.

Materials and Methods

Frozen Surimi Frozen surimi of Alaska pollack (SA grade) was kindly donated by Nissui Co. Ltd. (Tokyo, Japan). The surimi contained 4% sucrose and sorbitol, and 0.25% polyphosphate. The surimi was stored at -40°C until use.

Salted surimi preparation and its heating Frozen surimi stored at -40°C was transferred to a cold room (4°C) prior to use. Diced surimi was chopped in a food processor (Speed Cutter MK-K7, Matsushita Electric Industrial Co., Ltd., Osaka, Japan). To the surimi, 25% cold water was added together with 2.5% NaCl (W/W). The mixture was further chopped eight times for 30 sec with 30 sec intervals to make a homogeneous salted surimi paste. The paste was stuffed into aluminum pipes (ϕ 1.7 × 2 cm) and wrapped with polyvinylidene film. The samples were incubated at various temperatures in a water-bath to prepare preheated-gel. Another preheated surimi sample was immediately heated at 90°C for 30 min to produce two-step-heated gel. Both gels were cooled in ice-cold water to terminate the reactions.

Breaking force and breaking strain measurement of the thermal gel Breaking force and strain for both the preheated-gel and two-step-heated-gel were measured on a Rheometer RE-3305 (Yamaden, Tokyo, Japan) using a cylindrical plunger 3 mm in diameter. For the preheated-gel, measurement was done immediately after cooling to avoid additional changes in the physical properties by leaving at room temperature. Measurement for the two-step-heated-gel was routinely done after leaving it at room temperature for about 1 h as described elsewhere.

Analysis of myosin denaturation Incubated salted surimi was cooled in ice water. An aliquot (1–2 g) was homogenized immediately using a Polytron with 10 volumes of 50 mM NaCl, 20 mM Tris-maleate (pH 7.0) to reduce NaCl. As the salted surimi contained 2.5% NaCl, the final NaCl concentration was a little higher than 0.05 M, at which salt-induced myosin denaturation is negligible (Wakameda and Arai, 1984). The dilution also reduces the enzyme involved in the myosin cross-linking reaction. The homogenate was used for the analysis of myosin denaturation and cross-linking. The indices used for detecting myosin denaturation were Ca^{2+} -ATPase activity, salt solubility into 0.5 M KCl, and the solubility into 8 M urea. Ca^{2+} -ATPase was assayed in a medium containing 0.5 M KCl, 25 mM Tris-maleate (pH 7.0), 5 mM CaCl_2 , and 1 mM ATP at 25°C. For solubility measurement, the homogenate was re-suspended in a medium

containing either 0.5 M NaCl or 8 M urea containing 20 mM Tris-HCl (pH 7.5). The mixture was centrifuged to obtain the supernatants, which were referred to as salt-soluble and urea-soluble fractions, respectively. Solubility in the present study was defined as the amount of solubilized myosin into the above two media. The amount of myosin in the fractions was determined by measuring myosin heavy chain (MHC) content with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a SHIMADZU dual wavelength flying spot scanning densitometer CS-9300 PC (Shimadzu, Kyoto, Japan) (Konno and Imanura, 2000). Myosin content in the sample before centrifugation was determined by measuring the staining intensity of MHC in the whole samples dissolved in 8 M urea, 2% SDS, 2% 2-mercaptoethanol, and 20 mM Tris-HCl (pH 8.0) (SDSUM-solution) (Numakura *et al.*, 1985).

Analysis of myosin cross-linking Salted surimi, preheated at various temperatures, was dissolved in SDSUM-solution directly, and then applied to SDS-PAGE consisting of 3% polyacrylamide and 0.5% agarose (Konno *et al.*, 1998). The decrease in MHC content, as a result of cross-linking, was the index of the reaction.

All of the above experiments were repeated at least three times, and typical results were presented only when the same conclusion was obtained by the separate experiments.

Results and Discussion

Breaking force of the preheated and two-step-heated gels First, the breaking force of the preheated salted surimi, incubated at various temperatures, was measured. The breaking force of the two-step-heated gel at 90°C for 30 min was also measured to study the effect of preheating on improvements in the final gel properties. The temperatures employed were 15°C, 25°C, 35°C, and 45°C (Abe *et al.*, 1996). Both breaking force and strain were measured. As the increases in breaking force and strain profiles were similar (data not shown), only the change in the breaking force was presented (Fig. 1). When incubated at 15°C, salted surimi was still sol up to 120 min of incubation. The force gradually increased by prolonging the incubation time. Surimi incubated for 960 min showed a strength of about 200 g. As is well established, upon further heating of the preheated surimi for 30 min at 90°C, the breaking force increased drastically from 200 g to 1,200 g. The direct heating of salted surimi at 90°C for 30 min produced a gel with a breaking force of about 350–400 g. It was confirmed that pre-heating of salted surimi at 15°C increased the breaking force by heating at high temperature, and the setting effect was proven. Salted surimi incubated for less than 120 min was still sol, not gel. Nevertheless, heating of such pre-heated surimi significantly increased its breaking

Fig. 1. Increase in the breaking force of salted surimi upon incubation at various temperatures and further heating at high temperatures. Alaska pollack surimi with 2.5 % NaCl was incubated at 15°C (A), 25°C (B), 35°C (C), and 45°C (D), and further heated at 90°C for 30 min. The breaking force for the set gel (open symbols) and set-heated gel (closed symbols) was measured.

force. It was concluded that gel formation by preheating is not essential for achieving the setting effect.

When the incubation temperature was raised to 25°C, the breaking force for the preheated-gel reached maximum at around 180 to 240 min; the force observed was about 400 g. The breaking force of the preheated-gel increased gradually from the beginning of heating at 25°C without delay, which differed slightly from that observed at 15°C. Further heating at 90°C increased the force to 1,200 g, similar to that obtained at 15°C. Thus, the improving effect of preheating on breaking force development was similarly observed at 25°C as at 15°C.

Increases in the breaking force profiles for preheated and two-step-heated gels upon pre-heating at 35°C were also similar to those at 25°C, except that the time required to reach the maximal force was much shorter. The maximal force achieved by pre-heating for 60 min for the preheated-gel was about 400 g, which was the same as obtained at 25°C. The force for the two-step-heated gel was 1,000 g, which was slightly lower than that at 25°C.

The profiles at 45°C were completely different from those observed at lower temperatures. The obtained profiles confirmed previous results (Abe *et al.*, 1996). Incubation for 30 min formed a set gel showing a maximal breaking force of about 200 g, which subsequently decreased upon further

incubation. The force for the preheated gel incubated for 120 min was less than 100 g. Moreover, there was no increase in the force by further heating at 90°C. The force for the gel heated at 90°C (without preheating) showed a maximum breaking force of about 400 g.

Myosin cross-linking reaction in salted surimi It is reported that myosin forms cross-linked products when Alaska pollack salted surimi was incubated at relatively low temperatures (Numakura *et al.*, 1985; Kimura *et al.*, 1991). To analyze myosin cross-linking in salted surimi, incubated salted surimi was dissolved in SDSUM solution, as reported by Numakura *et al.* (1985). To increase the resolution of highly polymerized MHC, 3% polyacrylamide gel containing additional 0.5% agarose was employed. The patterns obtained are presented as (a) in Fig. 2A, B, C, and D. The gel system resolved MHC of progressively increasing polymerization, located above MHC monomer. MHC₂ (dimer) was the most abundant among the polymers, while the most highly polymerized MHC separated on the gel was MHC₅, migrating just below the connectin band (1000 kilodalton (kDa)) (Maruyama, 1986). The decrease in MHC content was the index of the progress of cross-linking, as measuring MHC is much simpler than measuring all of the polymerized MHC and because the amount of degraded fragment derived from MHC was small for the surimi used in this study. At 15°C

Fig. 2. Myosin cross-linking, as revealed by agarose/polyacrylamide gel, during the incubation of salted surimi, and the changes in salt and urea solubilities. The set gels in Fig. 1 were dissolved in SDSUM-solution directly for analysis of cross-linking reaction (a). The incubated surimi was homogenized in 10 vol of 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), and the soluble fractions in 0.5 M NaCl (c) and 8 M urea (b) were analyzed on the same gel. (A) to (D) incubation temperatures are as described in Fig. 1. HC and HC with numbers are myosin MHC and its cross-linked polymers. Act denotes actin band. 140 kDa and 80 kDa are the degraded fragments of MHC.

(Fig. 2A-a), cross-linked products appeared slowly with incubation. Although the surimi incubated for 360 min showed MHC₅, MHC was still abundant, indicating a slow progress of cross-linking at this temperature. Cross-linking became remarkable at 25°C, with a progressive decrease in MHC content and a production of cross-linked MHC polymers (Fig. 2B-a). Decrease in the MHC polymers (MHC₂₋₅) was accompanied by the production of components present at the top of the gel in the latter phase of incubation. The pattern for the samples incubated at 25°C for 15 min was similar to that at 15°C for 360 min, indicating a promoted reaction by raising the temperature from 15°C to 25°C. When incubated for 120 min, a slight amount of MHC degradation products (termed as 140 kDa and 80 kDa fragments migrating below MHC) was seen (Konno and Imamura, 2000). Similar progress in cross-linking was observed at 35°C, at which incubation for 10 min was long enough for the production of polymers, and protein present on the top of the gel increased with duration (Fig. 2C-a). However, the pattern at 45°C was different from the ones above. Although a slight amount of MHC₂ was detected during the very early phase, there was practically no progress in cross-linking (Fig. 2D-a). It was suggested that the enzyme responsible for the cross-linking reaction (transglutaminase) quickly lost its activity at this temperature.

Myosin aggregation during the preheating of salted surimi It is well known that heating of salted surimi causes myosin denaturation, namely the occurrence of conformational change. Such changes could not be detected by SDS-PAGE analysis. We investigated myosin denaturation in salted surimi by measuring its solubility in 0.5 M NaCl (salt-solubility) (Fig. 2-c) and in 8 M urea (urea-solubility) (Fig. 2-b). Loss of salt solubility is a well-known index of myosin denaturation. We also employed solubility in 8 M urea to obtain information on the state of aggregates formed. Aggregate formation is thought to be important in matrix formation in thermal gels. As we focused on the aggregate formation by myosin, we analyzed the SDS-PAGE pattern of the soluble fraction. The SDS-PSGE patterns are presented in Fig. 2 (b) and (c). SDS-PAGE patterns for the whole sample before fractionation are shown in Fig. 2 (a); the patterns for the samples dissolved in SDSUM-solution.

At 15°C, MHC recovered in the salt-soluble fraction decreased gradually with duration. By comparison of MHC content in (a), it was indicated that myosin aggregation proceeded slowly, even at a low temperature. In an early phase of incubation, a portion of MHC₂ was detectable in the salt-soluble fraction, but any MHC polymers higher than MHC₃ were not detected. MHC₂ is formed by cross-linking two MHC subunits, which constitutes the myosin molecule, and the product is intramolecularly cross-linked (Kunioka and

Ando, 1996). Thus, the MHC₂ detected in the fraction did not seem to be a member of the aggregated myosin. Moreover, a slight decrease of the actin band was detected for the sample incubated for a long period, such as 240 or 360 min. Thus, myosin in a form bound to F-actin formed aggregates. When the solvent was changed to urea from salt, high recovery of MHC and MHC₂ was obvious, showing mainly MHC and MHC₂. Although the majority of cross-linked polymers were not soluble, even in urea solution, a portion of the MHC polymers was still detected (Fig. 2A-b). It was suggested that cross-linked myosin formed rigid aggregates that do not disassemble with urea. As almost the entire actin band was recovered in the urea-soluble fraction, actin was not a member of the rigid aggregates.

When the temperature was raised to 25°C, the amount of MHC in the salt solution was remarkably low, and practically no MHC was detected for the sample incubated for 60 min. A very faint amount of MHC₂ was detected in the early phase, disappearing soon thereafter. Again, no MHC polymer higher than MHC₃ was detected in the salt-soluble fraction, as seen at 15°C. As the amount of MHC in the urea-soluble fraction (b) was less than that in the SDSUM fraction (a), a portion of the non-cross-linked MHC lost its solubility, especially in the latter phase of the reaction. A portion of the myosin polymers was recovered in the urea-soluble fraction, but the majority was insoluble. Loss of urea-solubility followed the loss of salt-solubility, which indicated a progression of myosin aggregation, from weak to rigid, with duration.

When incubated at 35°C, myosin rapidly lost salt-solubility, showing no MHC for the sample incubated only for 10 min. Similarly, the majority of MHC detected in the SDSUM fraction (a) incubated for 10 min had lost urea-solubility, which was slightly different from the events at 15°C and 25°C, where loss of urea-solubility did not occur as quickly. The result indicated that incubation at 35°C produced very rigid aggregates, through non-covalent bonds, that urea could not disassemble. Moreover, the majority of MHC polymers formed at 35°C were urea-insoluble. This fact also confirmed that aggregates formed at this temperature were more rigid than those formed at low temperatures. We noticed a high recovery of actin in the urea-soluble fraction, indicating that actin detached from myosin aggregates and was not involved in such rigid myosin aggregates.

When incubated at 45°C, an immediate loss of salt-solubility of myosin upon heating occurred. This is reasonable because a quick denaturation of myosin at that temperature was expected. A quite large amount of actin was detected in the fraction, indicating that myosin formed aggregates after detaching from actin. However, almost all MHC in the sample was recovered in the urea-soluble fraction. Thus,

myosin aggregation at this temperature was less rigid than at 35°C. In other words, myosin aggregation at 35°C and 45°C was clearly distinguished by the difference in urea-solubility. Differences in urea-solubility at 35°C and 45°C may be related to the presence or absence of cross-linked MHC. Cross-linking of MHC is the event whereby an isopeptide is formed between a γ -NH₂ group of Gln on one polypeptide chain and an ϵ -NH₃⁺ group of Lys on another peptide chain. The presence of a branched cross-linked polypeptide chain might trap MHC monomers in the aggregate structure non-covalently. Loss of urea-solubility was observed both at 25°C and 35°C. Decrease in MHC, namely the extent of cross-linking, at 25°C was greater than that at 35°C. Nevertheless, the loss of urea-solubility of MHC was less at 25°C. These results showed that loss of urea-solubility is not determined only by the extent of cross-linking. It is reasonable to think that rigid aggregate formation by myosin surely increases gel formation. The high breaking force of the preheated gels formed at 25°C and 35°C was due to rigid aggregate formation, together with cross-linking.

Ca²⁺-ATPase inactivation during the preheating process of salted surimi Ca²⁺-ATPase activity is a very sensitive index of myosin denaturation. To assess myosin denaturation in salted surimi, Ca²⁺-ATPase activity was assayed. Results are presented in Fig. 3. In the same figure, the loss of salt- and urea-solubility, and the progress of cross-linking, as monitored by the decrease in MHC content, are also presented for comparison. Ca²⁺-ATPase inactivation proceeded slowly at 15°C, showing one-half inactivation in 220 min. Inactivation progressed similarly to myosin cross-linking reaction, indicating that inactivated myosin readily formed cross-linked polymers. The loss of urea-solubility occurred faster than Ca²⁺-ATPase inactivation. Incubation for 240 min led to almost complete loss of urea-solubility, where the remaining activity was still about 42%. The changes clearly showed that ATPase-retaining myosin lost its salt- and urea-solubility, or aggregate formation was quicker than inactivation. Loss of salt-solubility was slightly faster than that of urea-solubility, and its half reduction occurred in 40 min, which was roughly five times faster than Ca²⁺-ATPase inactivation. This fact demonstrated that myosin aggregation, as measured by loss of solubility in salt and urea, was an earlier event occurring in the myosin molecule upon incubation of surimi than Ca²⁺-ATPase inactivation or cross-linking reaction. We have reported the preceding loss of salt-solubility to inactivation in carp myofibrils (Konno *et al.*, 2000). This phenomenon seems to be a general feature in the thermal denaturation of fish myofibrils or muscle. The same phenomenon was observed at 15°C, where a roughly parallel Ca²⁺-ATPase inactivation and progression of cross-linking were

Fig. 3. Loss of solubilities in salt and urea, ATPase inactivation, and myosin cross-linking upon incubation of salted surimi. ATPase inactivation (closed circles) was measured with surimi homogenates as prepared in Fig. 2. Solubilities in 0.5 M salt (open circles) and in 8 M urea (open squares) were calculated from the remaining MHC in the pattern in Fig. 2. Decrease in MHC content (open triangles) was also calculated from the pattern for the samples dissolved in SDSUM-solution as an index of myosin cross-linking reaction in Fig. 2. (A) to (D) were incubated at the same temperatures as described in Fig. 1.

produced. Moreover, the ATPase inactivation profile was quite similar to that of the increase in the breaking force. The maximal breaking force was obtained in 600 min, at which point almost complete inactivation was achieved. Faster loss of salt- and urea-solubility than Ca²⁺-ATPase inactivation and cross-linking were phenomena seen at both 15°C and 25°C. The one-half decrease in urea-solubility and Ca²⁺-ATPase inactivation was obtained in 20 min and 40 min, respectively. A similar progress in ATPase inactivation and breaking force increment was again observed at this temperature. At 35°C, Ca²⁺-ATPase inactivation occurred too quickly to follow and no activity was detected for the sample incubated for 10 min (the shortest time for analysis). Loss of salt-solubility was also completed in 10 min. Loss of urea-solubility also progressed quickly and a complete loss was achieved in 30 min. Myosin cross-linking reaction, monitored by the decrease in MHC content, was the slowest event among the indicators

used. Therefore, myosin cross-linking reaction at 35°C was an event occurring after a complete inactivation of myosin, which was completely different from the event at 15°C and 25°C. Irrespective of the activity, cross-linking took place with aggregated myosin. Events at 45°C were dissimilar to ones at other temperatures. Reasonably, Ca²⁺-ATPase inactivation and loss of salt-solubility occurred too quickly to follow. As aggregation is a phenomenon accompanied by myosin denaturation, the rate is temperature dependent. However, the decrease in urea-solubility at 45°C was much slower than that at 35°C, and the solubility was kept high even after incubation for 60 min (roughly 50%).

These results clearly demonstrated that myosin denaturation is not a simple matter and is deeply dependent on the heating temperature. Furthermore, well-balanced myosin denaturation, as detected by the loss of urea-solubility together with other denaturation indices, is important to achieving the setting effect. For practical purposes, preheating at 35°C for a short time is favored to achieve the highest setting effect. As the suitable preheating temperature differs according to fish species, and is dependent on the thermal stability of the myosin, further research is needed to clarify the most suitable temperature for other species of fish.

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