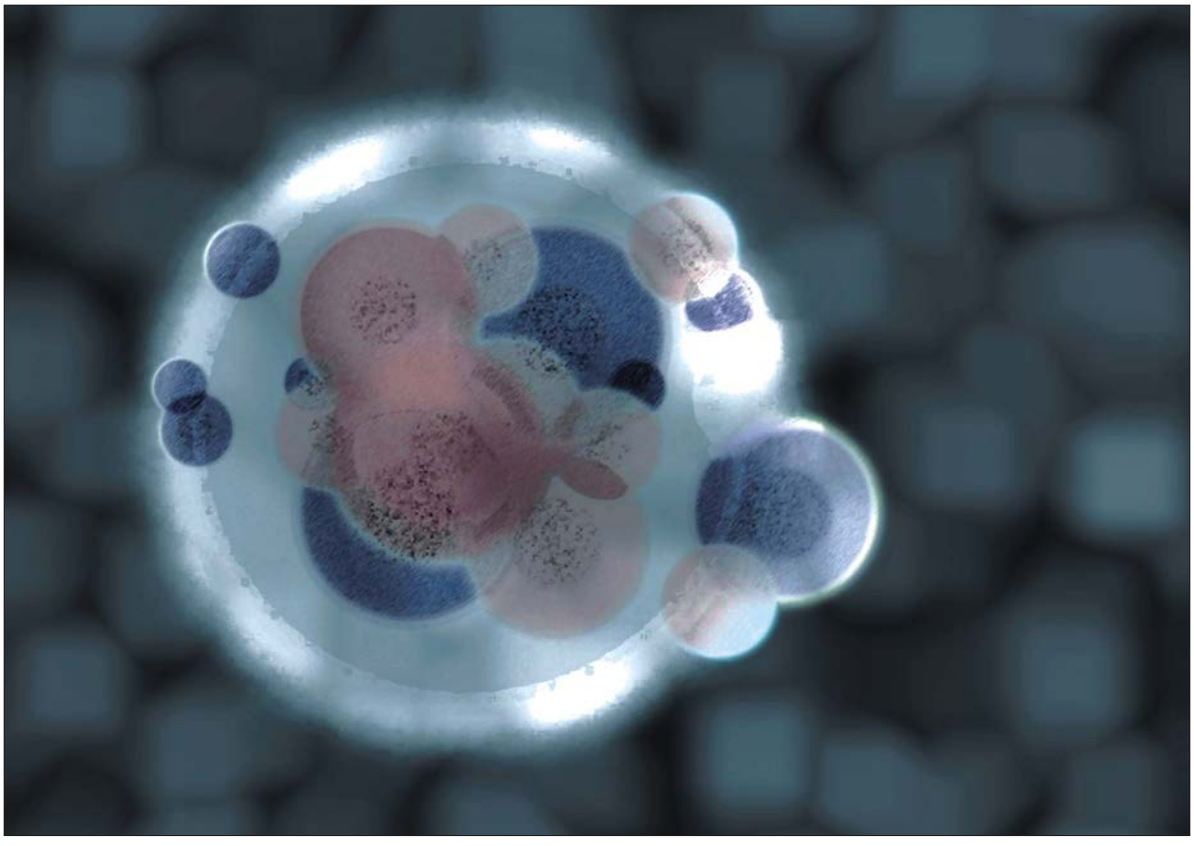

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 10 (5): 432-439, 2010
ISSN 1727-3048

Gel-forming Characteristics of Surimi from White Croaker under the Inhibition of the Polymerization and Degradation of Protein

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Abstract: The aim of this study is to investigate gel-forming characteristics of white croaker protein itself. This study was carried out under the inhibition of the polymerization and degradation of myosin heavy chain (MHC) by ethylenediaminetetraacetic acid and leupeptin. The heat-induced gels were prepared by preheating at temperatures in the range of 30-80°C for 20 min or 2 h and then heating at 80°C for 20 min and were evaluated in terms of gel strength. In order to investigate the behavior of proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out. Folin method was used to clarify the degradation of proteins by protease. Suwari occurred during preheating at 40 and 50°C and it contributed to gel-strengthening effect of the 2-step heating gels preheated at 50°C, indicating that non-covalent bonding reinforced the gel during setting and subsequent heating at 80°C even under the inhibition of the polymerization and degradation of MHC. The gel strength of 2-step heating gels was not affected by preheating temperature for 20 min, except for the gel preheated at 50°C under the inhibitory condition. Those gel strength values were similar to that of the gel heated directly at 80°C for 20 min. The maximum gel strength was obtained at 50°C. This suggests the gel-forming ability of white croaker protein itself. At temperature above 60°C, the gel strength decreased with preheating time, though the degradation of MHC was suppressed, indicating that non-proteolytic modori contributes to the weakening of gel of white croaker surimi.

Key words: White croaker surimi, gel-forming ability, polymerization, degradation, myosin heavy chain

INTRODUCTION

Gel-forming ability of surimi is the most important functional requirement imposing good quality on surimi-based products (Saeki *et al*., 1995). The gel formation of surimi by heating at low temperature is called setting (suwari) that plays a major role in strengthening surimi gel. It was generally accepted that setting process closely correlated with the polymerization of myosin heavy chain (MHC) by transglutaminase (TGase), a Ca-dependent and sulfhydryl (SH) enzyme (Wan *et al*., 1994). On the other hand, the polymerization at high temperature was promoted by the oxidation of SH groups in the presence of oxidants or metal ions (Itoh *et al*., 1979; Kishi *et al*., 1995). Funatsu *et al*. (1993) and Yasunaga *et al*. (1996) found that gel strength increased markedly by subsequent heating at 90°C without any progression in the cross-linking reaction of MHC. Accordingly, they presented the hypothesis that the major forces between myofibrillar proteins in the two-step heated gel were non-covalent bonding, such as hydrophobic interactions. Thus, the formation of both covalent and non-covalent bonding between proteins, accompanied by the heating of salt-ground surimi, is important for determining the quality of two-step heated gel.

Besides that, a remarkable loss of elasticity of heat-induced gels occurred at temperature ranges of 50-70°C, leading to an impairment of the textural quality of surimi-based product. The gel degradation, so-called modori, is induced by endogenous heat-activated proteases, which degrade myofibrillar proteins. The proteolytic disintegration of myofibrillar proteins, which affects a number of properties such as gelation, water holing capacity and emulsification, results in the gel-weakening of surimi. The gel degradation varies with species, but is generally caused by two types of proteases, serine-type and cysteine-type proteases (Makinodan *et al*., 1987; Yanagihara *et al*., 1991; Cao *et al*., 1999; Ohkubo *et al*., 2005).

Those characteristics of gel formation of fish meats, including the specific properties of proteins, such as the polymerization of MHC by TGase cross-linking and

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disulfide bonding and the degradation of MHC by the proteases, are important in the manufacture of surimi-based products.

White croaker *Argyrosomus argentatus* is an important commercial bottom fish. White croaker surimi is well-known for the ability to form a strong, highly elastic gel and has been applied to the production of high-quality kamaboko. It was reported that the setting of the paste from white croaker proceeded at moderate rate compared with Alaska pollack and tilapia; white croaker possessed a great gel-strength (Shimizu *et al*., 1981; Katoh *et al*., 1984). In addition, although the protease activity in white croaker meat was very strong, white croaker surimi can form a strong, highly elastic gel. Furthermore, myofibrillar proteins make a bigger contribution to the setting than the activity of TGase (Tsukamasa and Shimizu, 1991; Araki and Seki, 1993). Therefore, if the gel formation could be investigated without the polymer and degradation of protein during heating process, the gel-forming ability and characteristic of myofibrillar protein itself of white croaker surimi could be understood clearer.

For this reason, our objective was to elucidate the gel-forming characteristics of white croaker protein itself under the inhibition of the polymerization and the degradation of MHC. In this study, EDTA and leupeptin were used to inhibit the polymerization and the degradation of MHC. EDTA inhibited the polymerization by chelating metal ion and calcium ion, meanwhile leupeptin inhibited both serine-type and cysteine-type protease of the MHC degradation.

MATERIALS AND METHODS

Materials: White croaker surimi (SA grade) was imported from Pacific Fish Processing Co. Ltd. (Thailand) by Maruha-Nichiro Co.Ltd (Japan). Samples were parked in a box and transported to the Aquatic Product Utilization laboratory, Kochi University (Japan) within 30 min. Frozen surimi was vacuum-parked and kept at -55°C until use.

Chemical reagents: EDTA was obtained from Dojindo (Kumamoto, Japan). Leupeptin was purchased from Peptide Institute (Osaka, Japan). All chemicals were of analytical grade.

Gel preparation: White croaker surimi was thawed at 5°C overnight in cold room. Then, surimi was adjusted to a moisture content of 80% and mixed with 3% NaCl for 4 min by mixer (Model MK-K48, National, Japan) in the presence or absence of the inhibitors, EDTA and leupeptin. The resulting pastes were stuffed into stainless steel cases (3.1 cm in diameter, 3.0 cm in height), wrapped in polyvinylidene chloride film. Two sets of heat-induced gels (1-step heating gels and 2-step heating gels) was prepared by the following methods: the 1-step heating gels were prepared by incubating the salted surimi paste in a water-bath at temperatures in the range of 30-80°C at 10°C intervals for 20 min or 2 h. The 2-step heating gels were also prepared from the 1-step heating gels, followed by further heating at 80°C for 20 min. The heated gels were cooled immediately in ice water and were stored at 5°C.

Measurement of gel strength: Gel strength (g cm^{-2}) was assessed by multiplying breaking strength (g cm^{-2}) and elongation that were measured by a rheometer (Model CR-200D, Sun Scientific Co. Ltd, Tokyo, Japan) according to the method of Shimizu *et al*. (1981). For each sample, five tests were conducted. The mean value and standard deviation of results obtained were calculated.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The heated gel (0.1 g) was homogenized with 4.5 mL of 0.05 M sodium phosphate buffer (pH 7.2) containing 8 M urea, 2% SDS and 0.5 mL of 0.09% N-ethylmaleimide (NEM) and then heated in boiling water for 2 min to make unreduced sample. To make reduced sample, 2-mercaptoethanol was added to final concentration of 10%. An aliquot of 10 µL from each sample was subjected to SDS-PAGE using 3% polyacrylamide gel according to the method of Weber and Osborn (1969).

Autolysis: Three grams of the 2-step heating gel were homogenized with 15 mL of 5% trichloroacetic acid (TCA) solution at 10,000 rpm for 5 min using an Ace Homogenizer (Nihon Seiki Kaisha Ltd., Tokyo, Japan). The filtrate of the resulting homogenate was used as an extract. TCA-soluble peptides were measured according to the Lowry method (Lowry *et al*., 1951). Tyrosine (Tyr) was used as a standard. The amount of TCA-soluble amino acids and peptides was expressed as µg Tyr-equivalent/g sample.

RESULTS

Temperature-gelation curves of white croaker surimi in the presence or absence of inhibitors: The changes in gel strength of white croaker surimi in the presence or absence of inhibitors at different preheating temperature of the 1-step heating and 2-step heating were shown in Fig. 1a-d. In the gel without inhibitors (control), white croaker surimi had the highest gel strength at 50°C in the 1-step heating and at 40°C in the 2-step heating with

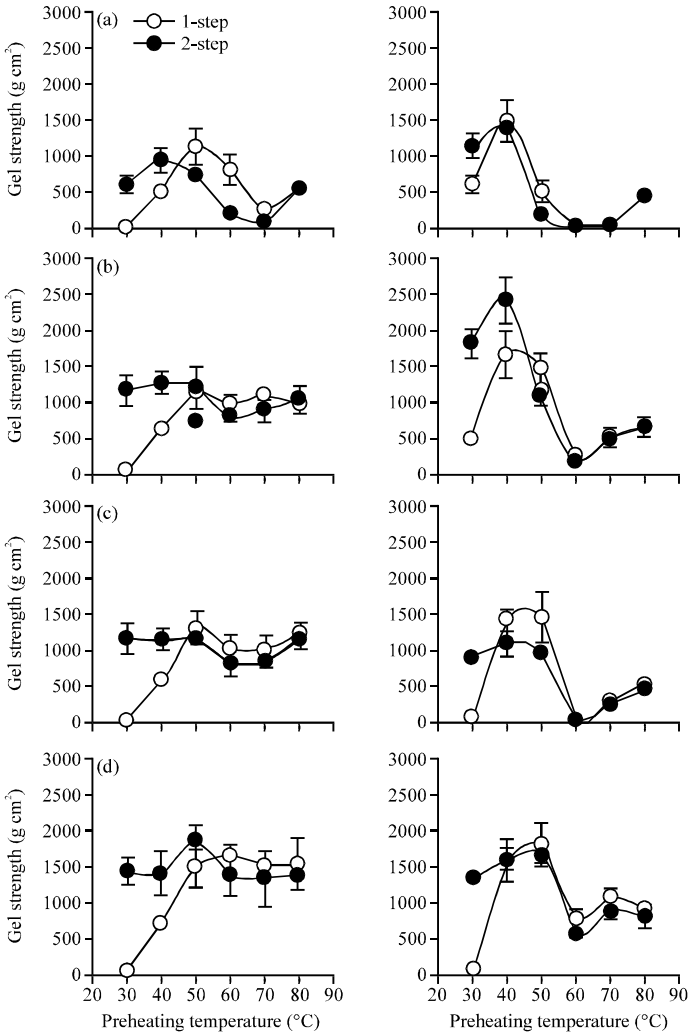

Fig. 1: Temperature-gelation curves of white croaker surimi in the presence or absence of inhibitors. Control, sample without inhibitors; 1-step heating at 30-80°C for 20 min (Left column) or 2 h (Right column); 2-step heating at 80°C for 20 min after preheating at 30-80°C for 20 min or 2 h, heating. (a) Control, (b) Leupeptin, (c) EDTA and (d) EDTA+Leupeptin

20 min preheating. The gel strength of gels preheated at 30 and 40°C increased with prolonged heating time and reached the highest value at 40°C. The lowest gel strength was obtained at 60 and 70°C for 2 h, indicating that modori was strongest around these temperatures.

In a preliminary experiment, to inhibit proteases, leupeptin concentration of 100 mg kg^{-1} exposed the highest effect (data not shown). Therefore, the concentration of 100 mg leupeptin kg^{-1} was used throughout the experiment on the change of gel strength of surimi under the inhibition of MHC degradation. The gel strength of surimi gels preheated for 20 min was higher than control, especially at modori condition. In the presence of leupeptin, the suwari effect appeared strongest at 40°C when preheated for 2 h. The gel strength of gel preheated at 40°C was the highest (2400 g cm^{-2}), approximately 2.5-fold higher than that of the surimi gels without inhibitors.

With the addition of EDTA, the polymerization of MHC was inhibited completely at a level of 10 mmol kg^{-1} EDTA (data not shown). The gel strength of gels preheated for 20 min was similar to that of gels in the presence of leupeptin. The gel strength of the 1-step heating gels preheated at 40 and 50°C increased with prolonged heating time. However, gel strength of these gels decreased after second step heating and it was equal to that of the gel heated directly at 80°C for 20 min. That is, suwari effect did not appear differently from in the presence of leupeptin.

In the presence of EDTA and leupeptin, the gel strength of the 1-step heating gels preheated for 20 min increased when heating temperature increased up to 50°C and then it reached plateau. However, the gel strength of the 2-step heating gels was not affected by preheating temperature, except for the gel preheated at 50°C. Those gel strength values were almost similar to the gel strength of the gel heated directly at 80°C for 20 min (1,400 g cm^{-2}). At 50°C, the gel strength of 1,800 g cm^{-2}, was obtained. The 1-step heating gel at 30°C was very weak. It was noted that the gel strength of gel preheated at 40°C increased with the preheating time in both 1-step and 2-step heating gels; and it was approximately 1.3-fold higher than that of directly heated gel at 80°C. Preheating at temperature above 60°C resulting in decrement in the gel strength of both 1-step and 2-step heating gels.

SDS-PAGE: SDS-PAGE patterns of white croaker surimi gel preheated at various temperatures for 20 min, which manifest the behavior of protein, in the presence of the inhibitors were illustrated in Fig. 2. In the unreduced and reduced samples of the control, the intensity of MHC bands decreased after preheated at temperature above 30°C. The bands between MHC and actin (MHC-A) occurred in larger amount at 60 and 70°C. In the presence of leupeptin, the intensity of MHC bands was almost constant. The MHC-A bands only occurred slightly. The MHC-A bands of samples containing EDTA were also observed but less than those of the control at the same temperature. In the presence of EDTA and leupeptin, the similar intensity of MHC bands was observed regardless of preheating temperature. Neither the polymer nor the MHC-A bands was observed in the reduced samples. It

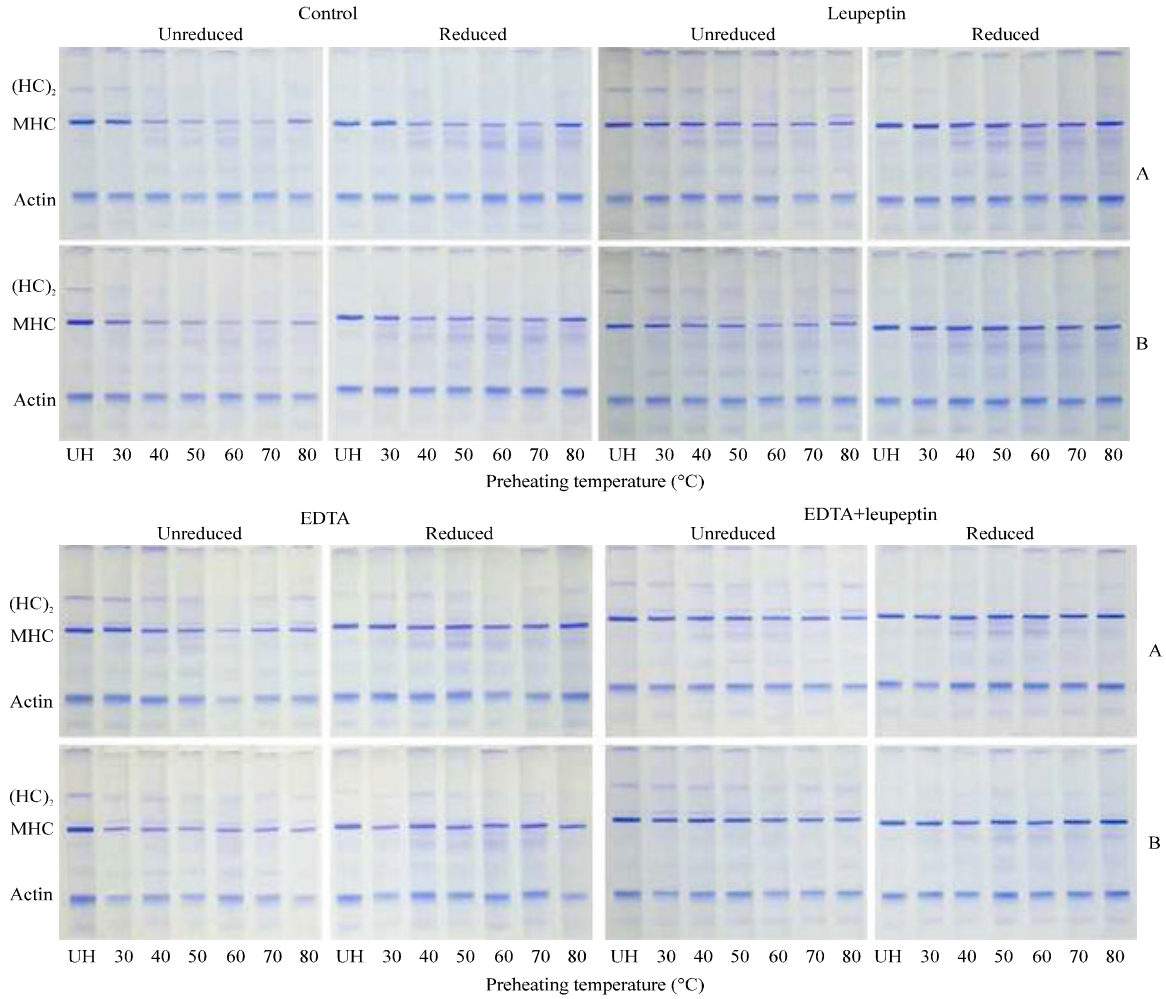

Fig. 2: SDS-PAGE patterns of reduced and unreduced samples of white croaker surimi gels in the presence or absence of inhibitors during setting for 20 min. A, 1-step heating at 30-80°C; B, 2-step heating at 80°C for 20 min after preheating at 30-80°C. MHC: Myosin heavy chain; $(HC)_2$: Dimmer of MHC; UH: Unheated surimi

indicated that preheating for 20 min, the polymerization and degradation of MHC was inhibited completely by EDTA and leupeptin.

SDS-PAGE patterns of white croaker surimi gel preheated at various temperatures for 2 h were showed in Fig. 3. In the unreduced and reduced samples of the control, the MHC bands of gel preheated in a range of 40-70°C almost disappeared after preheating for 2 h. This disappearance of MHC bands was accompanied by the occurrence of a large amount of MHC-A bands. The formation of polymer was observed at the pattern of gels preheated at 30 and 40°C for 2 h. In the presence of leupeptin, a larger amount of polymer was observed at the top of disc gels in the reduced samples of gels preheated at 30 and 40°C. In addition, the intensity of MHC-A bands in the reduced samples of gels preheated at 50-70°C was much less than those of the control, accompanying the more remaining of MHC. In the presence of EDTA, the intensity of polymer of heated gels was almost similar to that of the unheated sample and MHC-A bands were less than those of the control. The SDS-PAGE pattern of samples containing both EDTA and leupeptin showed the similar intensity of MHC bands regardless of preheating temperature. Neither the polymer nor the MHC-A bands were observed in the reduced samples even preheating for long time. Thus, these results indicated that EDTA and leupeptin inhibited both the polymerization and degradation of MHC.

Autolysis of white croaker surimi: To confirm the effect of inhibitors on the degradation of surimi proteins including MHC, the autolysis activity of white croaker

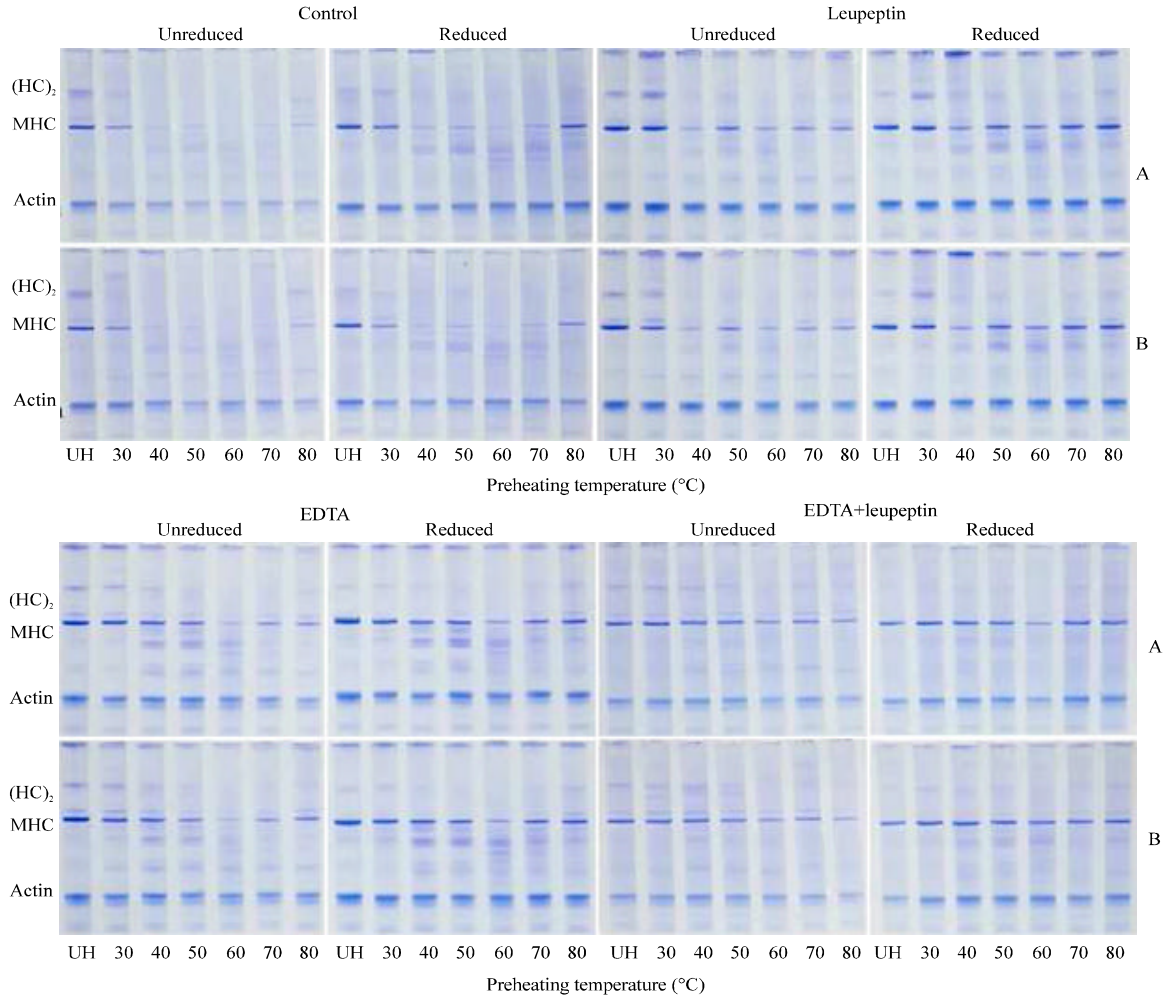

Fig. 3: SDS-PAGE patterns of the reduced and unreduced samples of white croaker surimi gels in the presence or absence of inhibitors during setting for 2 h. A, 1-step heating at 30-80°C; B, 2-step heating at 80°C for 20 min after preheating at 30-80°C. MHC: Myosin heavy chain; $(HC)_2$: Dimmer of MHC; UH: Unheated surimi

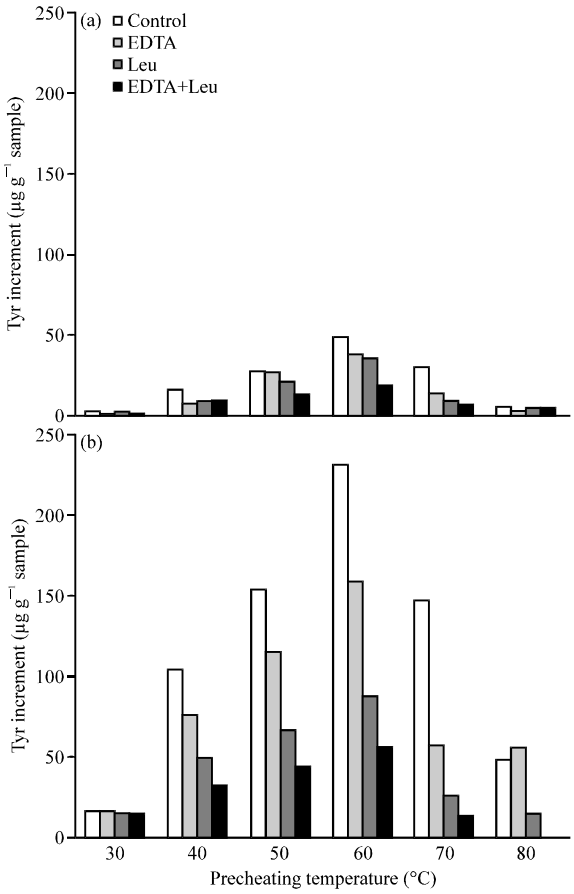

Fig. 4: TCA-soluble free amino acid and peptide in white croaker surimi in the presence or absence of inhibitors during 2-step heating. Preheating at 30-80°C for (a) 20 min or (b) 2 h, followed by heating at 80°C for 20 min

surimi at different temperatures and times was examined and was represented in terms of increment of Tyr (Fig. 4a, b). The increment of Tyr (μg g^{-1} sample) was defined as the difference between amount of TCA-soluble amino acid and peptide (TCA-soluble) of heated gel and that of unheated sample. TCA-soluble in white croaker surimi increased as temperature increased and reached the maximum value at 60°C. Subsequently, TCA-soluble decreased markedly during preheating at temperature above 70°C. TCA-soluble of samples containing EDTA was less than that of the control. By adding leupeptin, the production of TCA-soluble decreased more. The TCA-soluble content of gel preheating at 60°C for 2 h decreased 70 % amount as compared with the control. In the presence of EDTA and leupeptin, TCA-soluble decreased markedly. At the temperature of 70 and 80°C, a protease activity was almost inhibited. These results indicated that EDTA and leupeptin inhibited protease effectively.

DISCUSSION

In the addition of leupeptin, the gel strength of gel preheated at 40°C for 2 h was markedly higher than that of the control. The gel strength of the 2-step heating gel was remarkably higher than that of the 1-step heating gel. In addition, the polymers were formed in larger amount accompanied with less MHC-A substances. Furthermore, the production of TCA-soluble was suppressed. These results indicated that the competition between the polymerization and degradation of MHC during setting affected the gel-forming ability. These results agreed with Itoh *et al*. (1997) that the gel-forming ability at 40°C was related to both the depression of the polymerization and the enhancement of degradation of MHC to peptide. Also, Seki (1996) and Takeda and Seki (1996) reported that texture properties of kamaboko gels were affected by the proteolysis as well as the cross-linking of myosin during heating.

The gel strength of gels in the presence of EDTA was markedly higher than that of the control gel containing no inhibitors, especially at modori condition. Furthermore, EDTA suppressed the TCA-soluble. These confirmed that EDTA inhibited protease. It was reported that EDTA can inhibit metalo protease by chelating metal ion (Yongsawatdigul and Piyadhammaviboon, 2004) and calpain by chelating calcium ion (Barrett and Kirschke, 1981; Bond and Butler, 1987). The gel strength of the 1-step heating gel increased with preheating time at 40 and 50°C. However, the second step heating resulted in the marked decrease in gel strength. These results reconfirmed that covalent cross-linking by TGase occurring during preheating was necessary to strengthen the network structure (Kimura *et al*., 1991; Kumazawa *et al*., 1995), which was stabilized after second step heating at higher temperature, resulting in the formation of strong gel.

In the presence of EDTA and leupeptin, the gel strength of the 1-step heating gel was very weak when preheated at 30°C even for 2 h. However, the gel strength of the control or the gels containing leupeptin increased significantly at this temperature, indicating that the gel formation did not occur under the inhibition of MHC polymerization at 30°C. This result also confirmed that TGase plays a significant role in gel formation of white croaker surimi at 30°C. The gel strength of the 2-step heating gels was not affected by preheating temperature for 20 min, except for the gel preheated at 50°C under the inhibition of the polymerization and degradation of MHC. Those gel strength values were almost similar to that of the gel heated directly at 80°C for 20 min. In addition, the maximum gel strength was obtained at 50°C. This suggests the gel-forming ability of white croaker protein itself. Preheating at 40 and 50°C

proceeded the gel formation even under the inhibitory condition of the polymerization and degradation of MHC and it affected the gel formation of the 2-step heating gel. These results indicated that non-covalent bonding formed and reinforced the gel network during the setting and the second step heating. On other reports by Nowsad *et al*. (1994, 1996), suwari was also observed in Alaska pollack paste under the inhibition of the polymerization of MHC by TGase cross-linking, suggesting that non-convalent bondings play an important role at the setting around 40°C. However, our laboratory result showed that suwari occurred when preheated at low temperature in walleye pollock surimi, but after heated at 80°C for 20 min, the gel strength decreased and its value similar to that of the gel heated directly at 80°C for 20 min under the inhibition of the polymerization and degradation of MHC (Hossain *et al*., 2001). Similarly, a research about salmon surimi affirmed that when the polymer was not formed and the degradation of MHC was inhibited, the breaking strength of the 2-step heating gel was independent of the 1-step heating and it was also equal to that of gel heated directly at 90°C (Saeki *et al*., 1995). Thus, it was obvious that the gel formation under the inhibition of the polymerization and degradation of MHC of white croaker surimi was specific and differed from walleye pollack surimi.

The gel strength of gels heated at 70 and 80°C in the presence of EDTA and leupeptin decreased markedly with heating time, although the proteolysis of MHC was almost inhibited at these temperatures. It indicated that the gel was weakened by non-proteolytic modori at high temperature.

This study revealed the species-specific of white croaker surimi that suwari occurred during preheated at 40 and 50°C and the physical properties were kept or increased after heating at 80°C under the inhibition of the polymerization and degradation of protein. To assert these characteristics of white croaker surimi, the effect of preheating time under the inhibition of the polymerization and degradation should be carried out in the further study.

CONCLUSIONS

The gel-forming ability of white croaker protein itself could be evaluated by preheating for 20 min. The gel formation by non-covalent bonding during setting at 40 and 50°C contributed to gel-strengthening effect of the 2-step heating gels under the inhibition of the polymerization and degradation of MHC. This result might be a reasonable explanation for the high quality of white croaker surimi gel. The non-proteolytic modori contributed to the impairment of the gel of white croaker surimi at temperature above 60°C.

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