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Original Article

Inhibiting effect of polymerization and degradation of myosin heavy chain during preheating at 30°C and 50°C on the gel-forming ability of walleye pollack surimi

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ABSTRACT: To confirm the contribution of polymerization and degradation of myosin heavy chain (MHC) during preheating to the gel-forming ability of fish meat paste, walleye pollack surimi paste was preheated at 30°C and 50°C prior to heating at 80°C in the presence of various inhibitors. At 30°C, ethyleneglycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and ethylenediaminetetraacitic acid (EDTA) inhibited gel formation as well as the polymerization of MHC, whereas dithiothreitol (DTT) and leupeptin promoted gel formation, which was accompanied by the enhancement of MHC polymerization and decreased MHC degradation, respectively. At 50°C, leupeptin inhibited MHC degradation and improved gel strength, whereas EGTA, EDTA and DTT had no effect on MHC polymerization and degradation and did not affect gel formation. The results demonstrate that the gel strength of cooked gel (80°C) is not affected by preheating at 30°C and 50°C and does not inhibit polymerization and degradation. Results suggest that the gel strength of cooked gel is dependent on the polymerization and degradation of MHC during preheating.

KEY WORDS: **gel formation, modori, myosin heavy chain, oxidation, protease, suwari, transglutaminase, walleye pollack surimi.**

INTRODUCTION

In a previous paper, we reported that the increase and decrease in gel strength of cooked gel via preheating is related closely to the behavior of polymerization by non-disulfide covalent bonding and the degradation of myosin heavy chain (MHC), and that disulfide bonds seem to form when gel is cooked at 80°C and not during preheating at temperatures below 40°C.[1]

It is reported that this polymerization of MHC at low temperatures is because of the formation of MHC cross-linking[2–4] by an endogenous transglutaminase (TGase).[5,6] Tissue TGase is known to be a Ca-dependent and SH enzyme.

Another type of covalent bonding, intermolecular disulfide bonding, is a result of oxidation of sulfhydryl groups in the presence of oxidants or metal ions.[7–9] Therefore, this type of bonding is inhibited by a sulfhydryl group-blocking reagent such as *N*-ethylmaleimide,[10] iodoacetoamide,[11,12] or a metal ion chelating reagent such as ethylenediaminetetraacitic acid (EDTA).[9]

Alternatively, it has been reported that MHC degradation of walleye pollack surimi during heating is due to a serine-type and a cysteine-type protease because degradation is inhibited not only by serine protease inhibitors such as phenylmethylsulfonylfluoride (PMSF), *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK) and soybean trypsin inhibitor,[13] but also by cysteine protease inhibitors such as *N*-ethylmaleimide, iodoacetoamide[14] and E-64 (*N*-[*N*-(L-3-*trans* carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine).[15]

The purpose of the present experiment is to confirm whether preheating affects the gel-forming ability of cooked gel (at 80°C) by inhibiting the polymerization and degradation of MHC. Hence, the effects of various inhibitors on the poly-

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merization and degradation of MHC; that is, the effects of inhibitors against TGase, protease and sulfhydryl oxidation on the gel-forming ability of walleye pollack surimi, were investigated. Preheating was carried out at 30°C and 50°C, temperatures which have the strongest effect on the characteristics of suwari and modori, respectively, as shown in a previous paper.[1] SS and C grade surimi were used because SS grade is strongest in suwari and C grade is strongest in modori.[1]

MATERIALS AND METHODS

Materials

As in a previous paper,[1] we used unsalted SS grade (Maruha Co. Ltd, Tokyo, Japan) and C grade (Hirose Suisan Co. Ltd, Mombetsu, Hokkaido, Japan) walleye pollack frozen surimi.

Chemical reagents

In order to inhibit the polymerization and degradation of MHC, several kinds of chemical reagents were used. Table 1 shows the functional characteristics of the chemical reagents and the quantity that was applied to each surimi paste.

We applied 10 mmol of EGTA (Ca chelating reagent) per kg of meat to inhibit polymerization by TGase. An equal amount of EDTA (dication chelating reagent) was used to inhibit crosslinking polymerization by TGase and disulfide bonding through oxidation of sulfhydryl groups. We used 10 mmol DTT (reducing reagent) per kg of meat to inhibit the oxidation of sulfhydryl groups. To inhibit proteolysis by serine- and/or cysteine-type protease, 400 mg of leupeptin per kg of meat was applied.

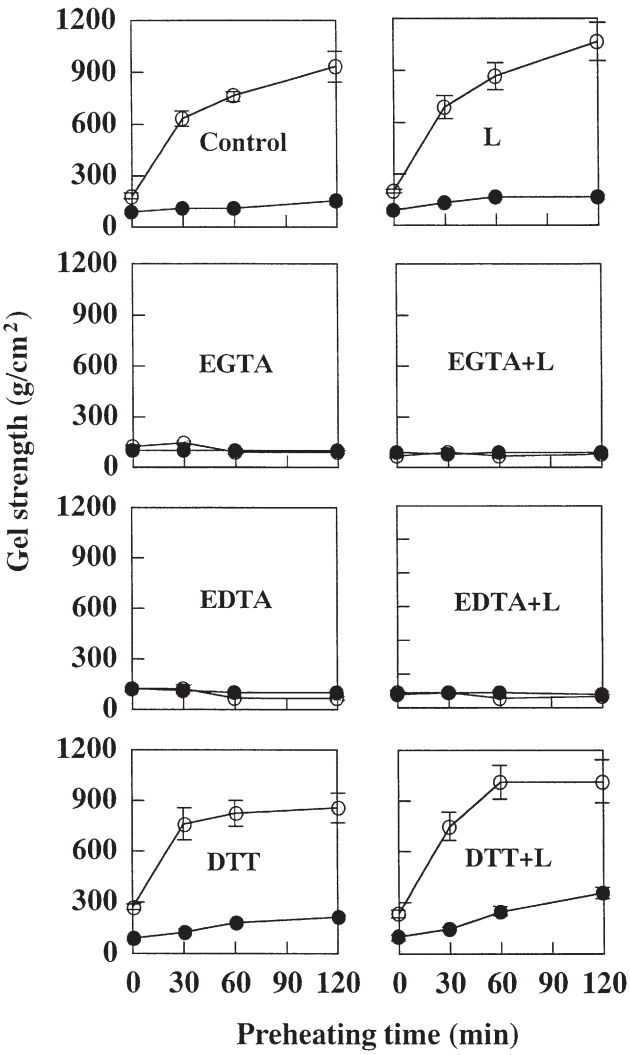

Fig. 1 Changes in gel strength of (○) SS and (●) C grade walleye pollack surimi gels preheated at 30°C for various periods prior to heating at 80°C for 20 min with various inhibitors. L, leupeptin.

Table 1 Functional characteristics of the inhibitors and their quantity applied in the surimi paste.

Inhibitor	Concentration (per kg meat)	Oxidation of SH	Cross-linking by TGase	Degradation by protease
EGTA	10 mmol	–	↓	–
EDTA	10 mmol	↓	↓	–
DTT	10 mmol	↓	↑	↑ (for SH protease)
Leupeptin	400 mg	–	–	↓
EGTA + leupeptin	10 mmol + 400 mg	–	↓	↓
EDTA + leupeptin	10 mmol + 400 mg	↓	↓	↓
DTT + leupeptin	10 mmol + 400 mg	↓	↑	↓

(↓) Depressing effect; (↑) promoting effect; (—) no effect.

TGase, transglutaminase; EGTA, ethyleneglycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacitic acid; DTT, dithiothreitol.

EDTA and EGTA were obtained from Dojindo (Kumamoto, Japan), DTT was obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan) and leupeptin was obtained from Peptide Institute (Osaka, Japan).

Proximate composition analysis

The protein and moisture contents of surimi were determined according to a method described elsewhere.[1]

Gel preparation

Unsalted walleye pollack surimi was thawed at 4°C overnight in the cool room. Surimi was adjusted to 80% in moisture content and ground together with various inhibitors (EDTA, EGTA and DTT 10 mmol/kg of meat each; leupeptin 400 mg/kg of meat) and 3% NaCl for 20 min. The resulting paste was stuffed into stainless steel cylinder cases (3.1 cm in diameter, 3.0 cm in height), wrapped in polyvinylidene chloride film and then heated in a water bath at 30°C and 50°C for 30, 60 and 120 min prior to heating at 80°C for 20 min. Then the gels were cooled immediately in ice water. Samples were stored at 4°C until required for assessing the gel properties.

Gel strength measurement

Gel strength was assessed by conducting a stretching test according to the method of Shimizu *et al.*[16] as described previously.[1]

Sodium dodecylsulfate–polyacrylamide gel electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Weber and Osborn[17] as described previously.[1]

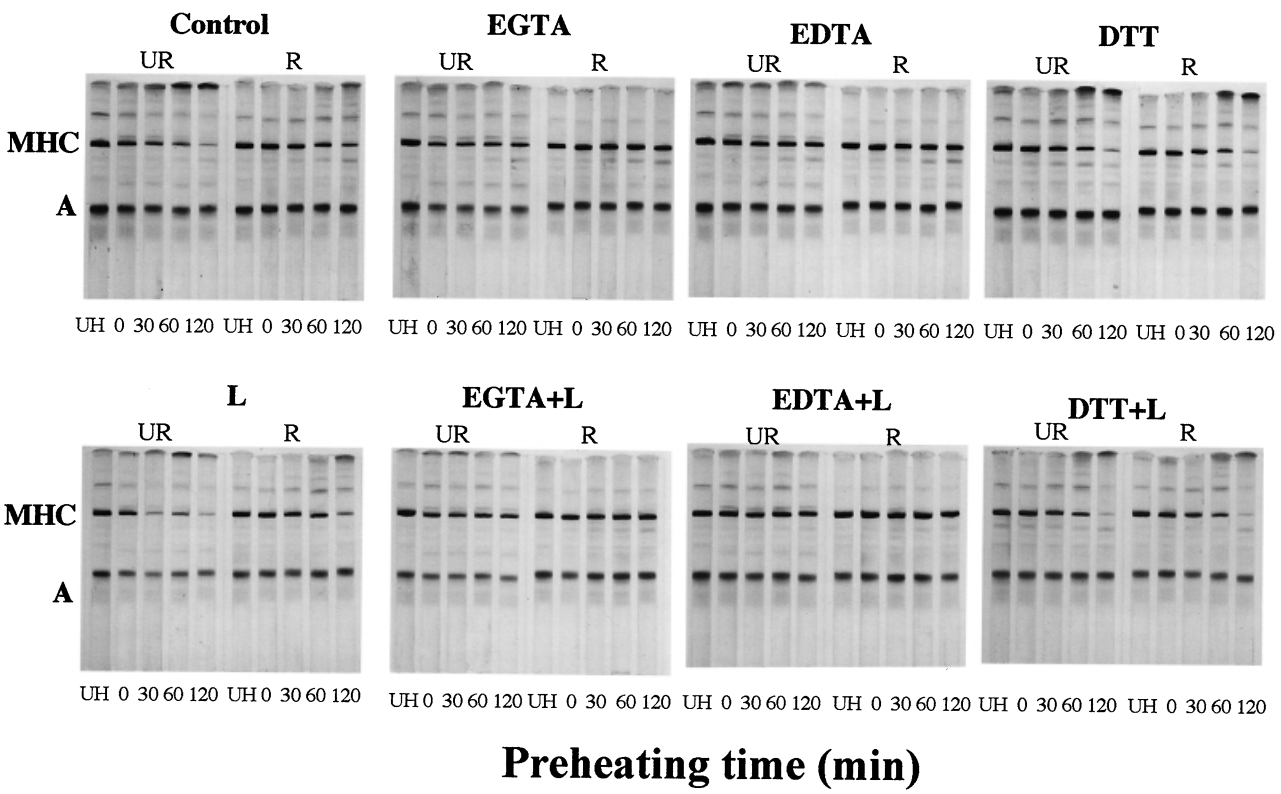

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of SS grade walleye pollack surimi gels preheated at 30°C for various periods before being heated at 80°C for 20 min with various inhibitors. MHC, myosin heavy chain; A, actin; UR, unreduced samples; R, reduced samples.

RESULTS AND DISCUSSION

Gel-forming ability of SS and C grade surimi at 30°C with various inhibitors

In order to examine the inhibiting effect of polymerization and degradation of MHC during preheating of surimi paste on gel formation, both surimi pastes (SS and C grade) were preheated at 30°C for 30, 60 and 120 min before being heated at 80°C with or without various inhibitors. The gel strengths of those gels are shown in Fig. 1. In control conditions without inhibitors, gel strength increased with increased period of preheating time. The increase in gel strength was much higher for SS grade than in C grade surimi. The addition of 10 mmol/kg of meat of EGTA and EDTA suppressed the gel formation process of both grades of surimi. It has been reported that 1 kg of walleye pollack surimi contains 2.76 ± 0.17 mmol/kg of calcium ions.[18] Wan *et al.* reported that the gel strength of salted meat paste did not increase during setting in the absence of calcium ions, and that the major effect calcium ions had on gelation was to activate intrinsic TGase.[18] Therefore, the inhibition of gel formation at 30°C by EGTA and EDTA indicates that intrinsic TGase was inhibited through the chelating Ca ions present in surimi.

The addition of DTT increased the gel strength of SS grade surimi gels preheated for 30 and 60 min, and that of C grade surimi gels preheated for 60 and 120 min. These results suggest that DTT enhances gel formation not by the oxidation of sulfhydryl groups to disulfide bonds but by other bonds, such as cross-linking by TGase, which is known to be a cysteine enzyme. It has been reported that reducing agensts have a similar effect on the setting of sardine paste.[19] Reducing reagents appear to promote the setting of fish meat paste.

Leupeptin increases gel strength, suggesting that proteolysis in surimi is inhibited when both DTT and leupeptin were added, gel strength increased to a maximum. This improvement in gel strength may be due to the simultaneous occurrence of the enhancement of TGase by DTT and the inhibition of proteolysis by leupeptin. It is worth noting that even in the presence of both DTT and leupeptin, the gel strength of C grade surimi did not reach that of SS grade. Usually fish used for C grade surimi is less fresh than that used for SS grade surimi. Katoh *et al.* reported that low-grade surimi shows low Ca-ATPase activity.[20] Recently, Seki *et al.*[21] reported that heat-denatured surimi shows a low gel formability and setting response. Therefore, myosin, the main protein that forms a gel network, in C grade surimi may be partly denatured more than myosin in SS grade surimi.

To investigate the behavior of protein molecules in each type of gel, SDS-PAGE was carried out. To differentiate polymerization that was caused by disulfide bonding from that caused by TGase cross-linking, unreduced and reduced samples of each gel were analyzed. Figures 2 and 3 show the SDS-PAGE patterns of SS grade surimi gels preheated at 30°C and the formation of polymers (larger-sized molecules than MHC) in reduced samples, which were estimated by densitometry, respectively. It

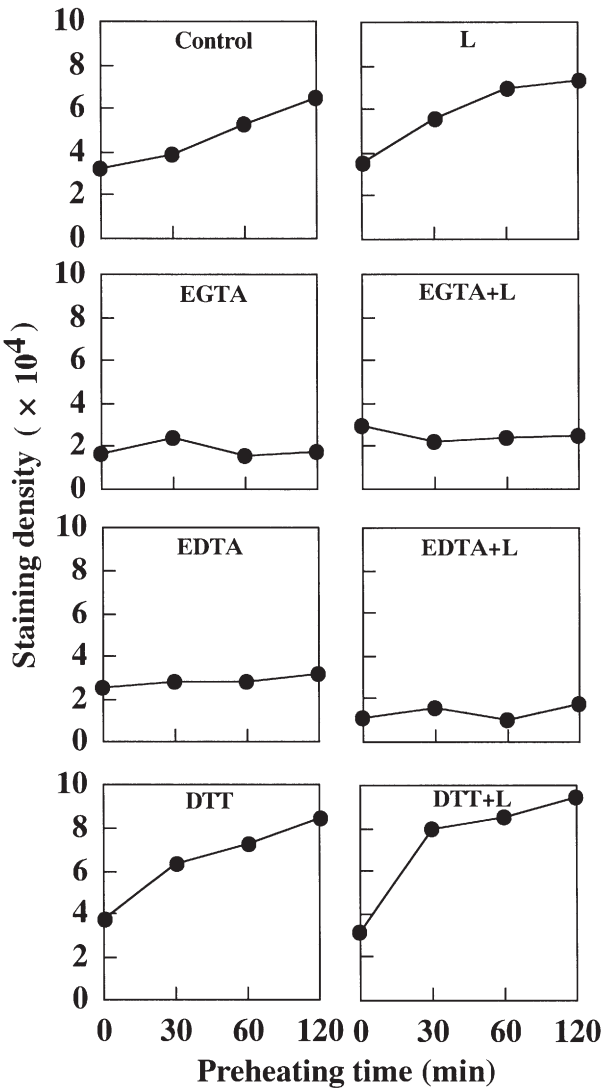

Fig. 3 Staining density of molecules bigger than myosin heavy chain (MHC) under reduced conditions in SS grade walleye pollack surimi gels preheated at 30°C for various periods before being heated at 80°C for 20 min.

was observed that the amount of MHC in control gel samples decreased proportionally with increasing polymer amounts in both unreduced and reduced samples with increased period of preheating time. The polymers observed in the reduced samples seemed to be formed through MHC cross-linking by TGase judging from the following results. In the presence of EGTA and EDTA, the amount of MHC was constant during preheating, indicating that cross-linking of MHC by TGase was inhibited through chelating calcium ions, which are essential for the activity. In the presence of DTT, polymer formation was promoted, suggesting that DTT activated TGase, which is an SH enzyme, during preheating at 30°C of walleye pollack surimi. The addition of DTT did not promote proteolysis, although a small quantity of substances were formed as observed between the MHC and actin bands (MHC-A substances) in the SDS-PAGE patterns of the reduced samples of control gel. In the presence of leupeptin, the formation of MHC-A substances was not observed and there was slightly more polymer formation compared with in gels without leupeptin, indicating that proteolysis of MHC and polymer formation was inhibited.

Figures 4 and 5 show the SDS-PAGE patterns of C grade surimi gels preheated at 30°C and the formation of polymers in the reduced samples, respectively. SDS-PAGE patterns (Fig. 4) of unreduced samples of the control gel (i.e. without any inhibitors) showed that MHC decreased with increased polymerization, but in reduced samples, increased polymerization was much less even in the 2 h setting compared with SS grade surimi (Figs 3 and 5). In the presence of EGTA and EDTA, the amount of MHC in the reduced samples appeared remain constant during preheating (Fig. 4) and polymerization did not increase (Fig. 5), indicating that MHC cross-linking by TGase was inhibited. In the presence of DTT, polymer formation was not promoted as much as in SS grade surimi (Fig. 5) and proteolysis was not enhanced (Fig. 4). In the presence of leupeptin, the formation of MHC-A substances was not observed in all gels and the formation of polymers was slightly increased. This indicates that MHC proteolysis and polymeriza-

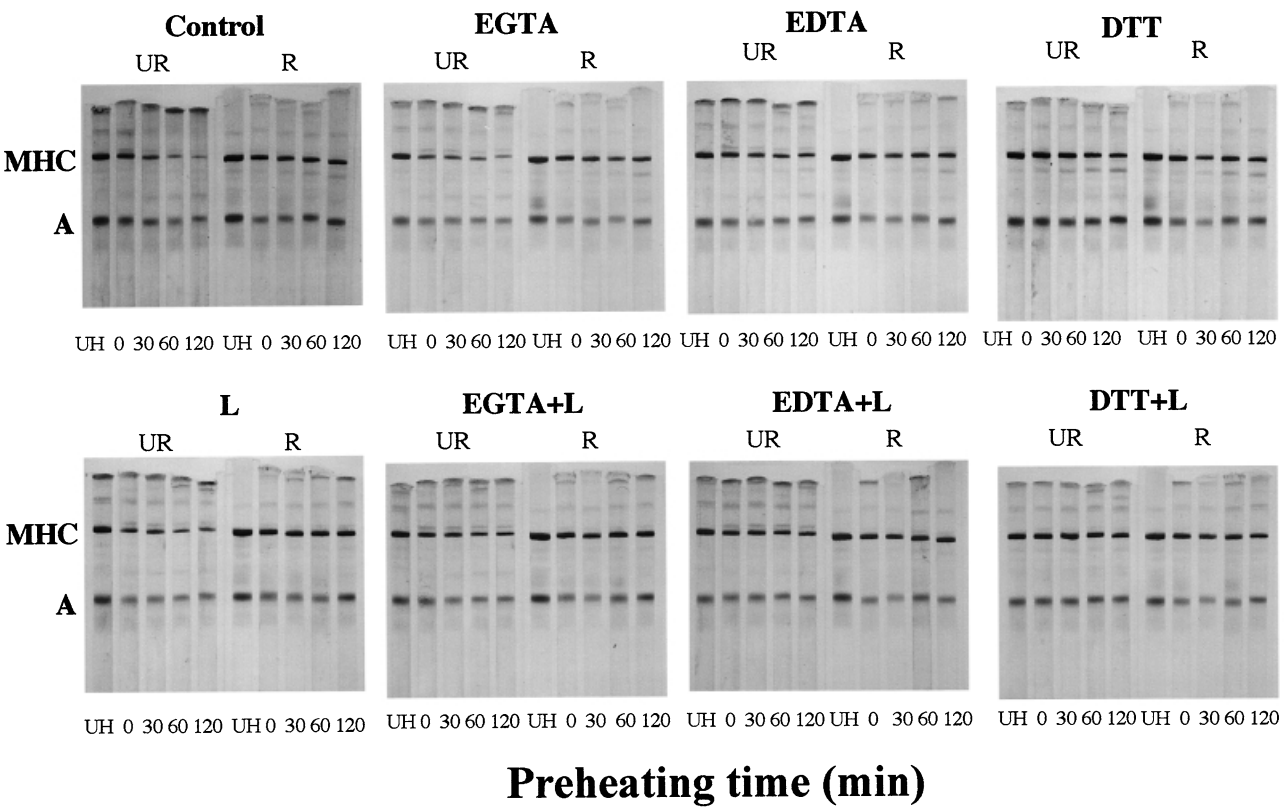

Fig. 4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of C grade walleye pollack surimi gels preheated at 30°C for various periods prior to heating at 80°C for 20 min with various inhibitors. MHC, myosin heavy chain; A, actin; UR, unreduced samples; R, reduced samples.

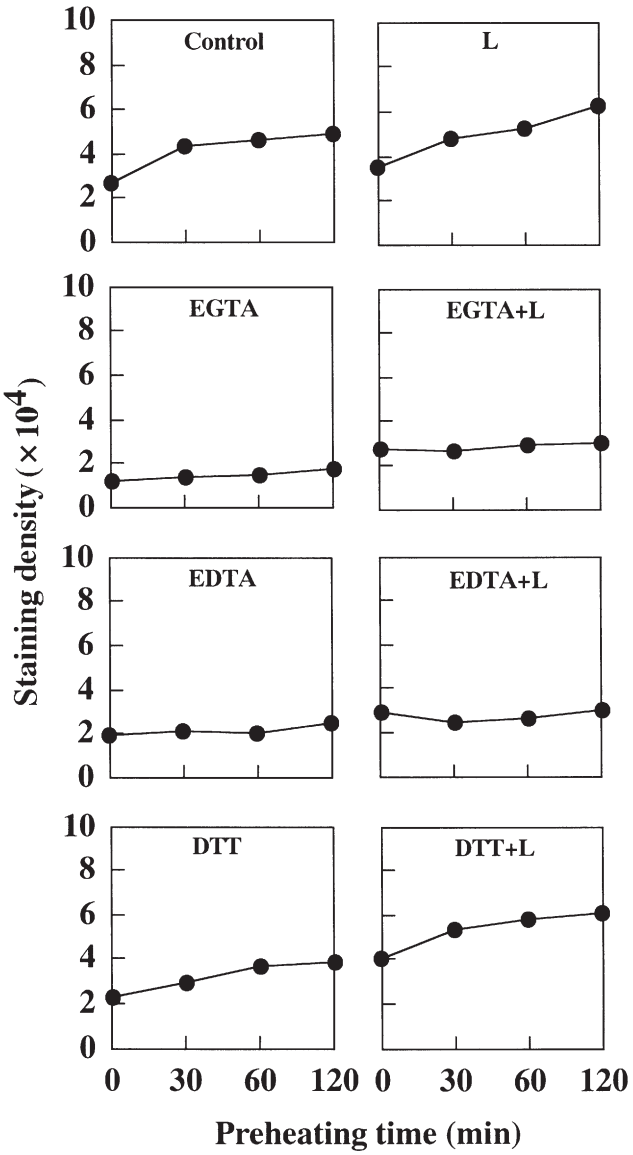

Fig. 5 Staining density of molecules bigger than myosin heavy chain (MHC) under reduced conditions in C grade walleye pollack surimi gels preheated at 30°C for various periods prior to heating at 80°C for 20 min.

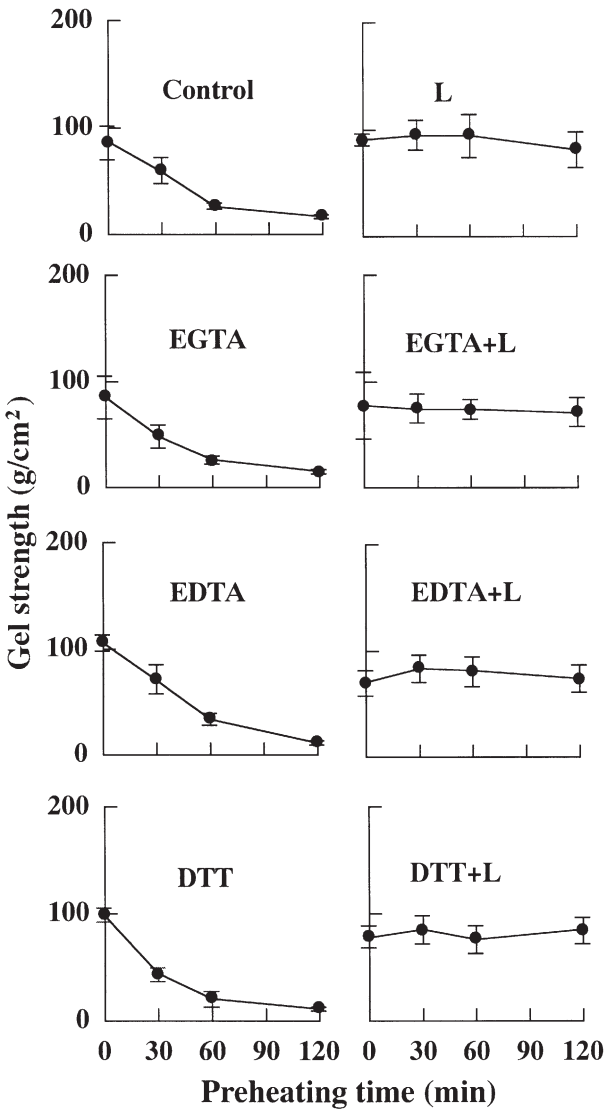

Fig. 6 Changes in gel strength of C grade walleye pollack surimi preheated at 50°C for various periods prior to heating at 80°C for 20 min with various inhibitors.

tion was also inhibited in C grade surimi. Even in the presence of DTT, an activator for TGase, and leupeptin, an inhibitor for degradation, polymers did not form in C grade surimi as much as in SS grade surimi, indicating that the polymerization ability of MHC induced by TGase is very weak in C grade surimi compared with SS grade. The results also suggest that TGase activity in C grade is not as strong as that in SS grade surimi, or that, as a substrate for TGase, myosin structure in C grade surimi is not as good as that in SS grade surimi. Seki *et al.* reported that denatured surimi has a weaker MHC polymerization ability than un-denatured surimi.[21]

From the aforementioned results, it was confirmed for both surimi that the increase in gel strength by preheating at 30°C depends on the polymer formation of MHC; that is, inhibiting polymerization does not increase gel strength during preheating and promoting polymerization enhances gel formation. Moreover, proteolysis lowers the contribution of polymerization by TGase to gel strength. Furthermore, it was found that gel strength is not affected by preheating time at 30°C if MHC polymerization and degradation are inhibited by adding their inhibitors.

However, Niwa *et al.* reported that suwari was

observed during the setting of meat paste even under conditions when TGase activity was inhibited and when the oxidation of sulfhydryl groups occurred.[22,23] In the present experiment, set gels heated at 35°C or 40°C were not heated again at 80°C or 90°C to cook. In other experiments, we are trying to compare gel strengths between one-step heating gels and two-step heating gels under conditions of inhibition of polymerization and degradation of MHC.

Gel forming ability of C grade surimi with various inhibitors at 50°C

In order to examine the effect of inhibiting proteolytic degradation during preheating on the gel strength of cooked gel, C grade surimi, which is strong in modori and MHC degradation,[1] was preheated at 50°C with and without various inhibitors. Changes in gel strength of C grade surimi paste via preheating are shown in Fig. 6. In the absence of inhibitors (control gel), gel strength gradually decreased with increased period in preheating time at 50°C prior to cooking at 80°C for 20 min. The addition of EGTA, EDTA and DTT did not affect gel strength. Leupeptin-added surimi gels showed no decrease in gel strength during preheating.

The SDS-PAGE patterns (Fig. 7) for the control gel at 50°C, show decreased amounts of MHC along with the formation of MHC-A substances in the unreduced samples and the absence of polymer formation in the reduced samples. This means that MHC degradation and almost no polymerization occurred during preheating at 50°C. In the presence of EGTA and EDTA, patterns almost similar to that of the control gel were observed in both unreduced and reduced samples, indicating that polymerization or degradation were not affected by these inhibitors at 50°C. In the control gel, the addition of DTT caused very little polymerization and the amount of MHC decreased proportionally with the production of MHC-A substances, indicating that DTT did not affect degradation. Leupeptin inhibited the decrease in MHC and the amount of MHC appeared to remain constant during preheating, suggesting that cysteine and/or serine protease is involved in proteolysis in

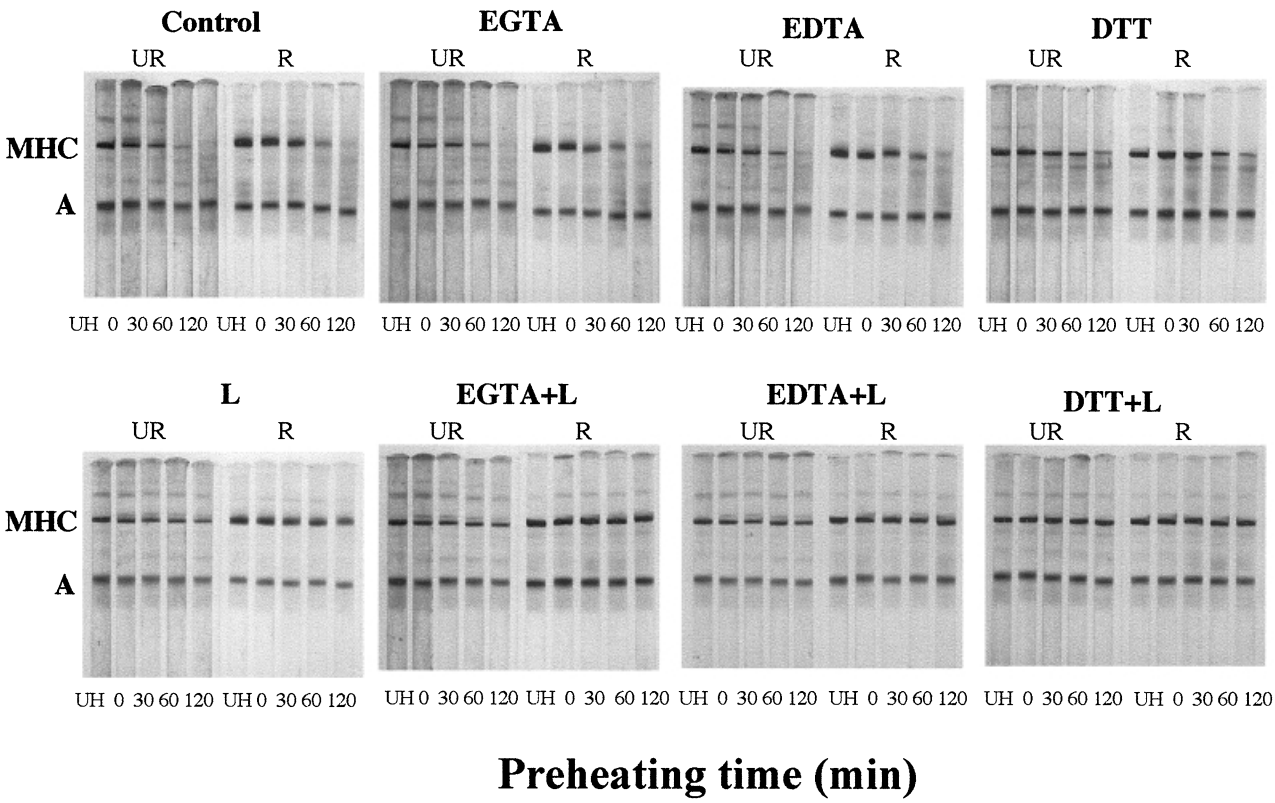

Fig. 7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of C grade walleye pollack surimi gels preheated at 50°C for various periods prior to heating at 80°C for 20 min with various inhibitors. MHC, myosin heavy chain; A, actin; UR, unreduced samples; R, reduced samples.

walleye pollack surimi gel, as reported by Liu *et al.*[13,14] and Takeda *et al.*[15]

From the results of gel strength and the SDS-PAGE patterns of gels preheated at 50°C, it was confirmed that decreased gel strength when preheating at 50°C is due to the proteolytic degradation of MHC. Furthermore, it was found that gel strength is not affected by preheating time at 50°C if MHC degradation is inhibited by adding protease inhibitor.

From the present experiments it was concluded that the gel strength of cooked gel is not affected by preheating time at 30°C and 50°C if MHC polymerization and degradation do not occur during preheating. These results suggest that the structural changes that occur in MHC in salted surimi and induced during preheating do not affect the gel strength of final cooked gel, especially if the structural changes are not accompanied by polymerization due to covalent bonding and degradation by proteolysis of MHC during preheating.

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