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Effect of $KBrO_3$ on Gel-forming Properties of Walleye Pollack Surimi through Setting with or without Transglutaminase Inhibitor

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Abstract: In order to clarify the effect of oxidation itself on the gel formation of salted-surimi through setting, the gel-forming properties were examined with or without transglutaminase (TGase) inhibitor. The gels were prepared from walleye pollack salted-surimi mixed with $KBrO_3$ through setting at 30°C (suwari gel) for 2 h prior to heating at 80°C for 20 min (kamaboko gel) in the presence or absence of TGase inhibitor. The gel strength of kamaboko gel increased through setting but $KBrO_3$ almost did not promote the gel formation of kamaboko gels through setting comparing with control. $KBrO_3$ increased the breaking strength and decreased elongation so that the gel became harder. Even in the presence of TGase inhibitor, where the increase in gel strength during setting was suppressed, $KBrO_3$ showed the promotion of gel formation, although the gel strength is lower than the gel without TGase inhibitor. Almost, similar behaviors of protein polymerization by disulfide bonds and the oxidation of sulfhydryl groups to those in the absence of TGase inhibitor were observed. These results suggest that $KBrO_3$ enhances the gel forming ability of walleye pollack surimi through the disulfide bonding of myosin heavy chain by oxidation during the setting. In conclusion, the oxidation of salted surimi sol during setting at 30°C can contribute to the gel formation, but its contribution seems not to be cooperative with that of TGase.

Key words: Gel forming ability, setting, oxidants, sulfhydryl groups, disulfide bond, pollack, surimi

INTRODUCTION

The gel physical property of fish surimi products like kamaboko is one of the important qualities. It is well accepted that setting or suwari plays a major role in strengthening surimi gels because of the polymerization of Myosin Heavy Chain (MHC) through ϵ-(γ-glutamyl) lysine cross-linking by TGase during setting, resulting in the enhancement of surimi gel quality (Benjakul *et al*., 2003; Gilleland *et al*., 1997; Kimura *et al*., 1991; Kumazawa *et al*., 1995). So, setting procedure is now very popular in surimi product processing.

Additionally, disulfide bond that is another predominant covalent bond contributive to the interaction of protein molecules constitutes a portion of the further development of the gel network during heating at high temperature (Lanier, 2000; Montero and Gómez-Guillén, 1996). The intermolecular disulfide bonding is a result of the oxidation of sulfhydryl groups in the presence of oxidants or metal ions (Itoh *et al*., 1979; Kishi *et al*., 1995). The addition of $KBrO_3$ has been shown to improve the strength of gels from fish proteins which otherwise had poor gelling ability (Okada and Nakayama, 1961). The mechanism of this improvement was generally accepted to be via oxidation of sulfhydryl compounds to disulfide bonds (Lee *et al*., 1997) and inactivation of proteinase during heat-setting (Pacheco-Aguilar and Crawford, 1994). Therefore, the gel formation of fish protein can be considered to be enhanced by the MHC polymerization by the oxidation of protein sulfhydryl groups to disulfide bonds caused by the effect of oxidants as well as by the cross-linking resulted from TGase during setting.

However, the effect of oxidation of surimi protein during setting on the gel formation has not been extensively studied. It is not sure whether oxidants may contribute to the promotion of gel formation synergetically or competitively with TGase, since TGase is known as a SH enzyme.

Therefore, the objective of this study was to investigate the effect of $KBrO_3$ on gel forming properties of walleye pollack surimi during setting at 30°C with or without a transglutaminase inhibitor.

MATERIALS AND METHODS

Chemical reagents: Potassium bromate ($KBrO_3$) was obtained from Wako Pure Chemical Industries Co. Ltd, (Osaka, Japan). Ethylene glycol-O,O'-bis(2-aminoethyl)-

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N,N,N',N'-tetraacetic acid (EGTA) was obtained from Dojindo (Kumamoto, Japan).

Gel preparation: Frozen walleye pollack *(Theragra chalcogramma)* surimi (SS grade, Maruha- Nichiro Co. Ltd. Japan) was used as a raw material. The surimi measured 76.6 and 14.2% in moisture and protein content, respectively. The surimi that was thawed by leaving at 5°C overnight was chopped for 1 min using a chopper (MK-K48 Matsushita, Japan). The chopped surimi was mixed with the mixture of chilled water (to adjust 80% moisture content), NaCl (3% against 80% moisture surimi) and $KBrO_3$ (0, 2 and 4 μmol g^{-1}) with or without 10 mM of EGTA for further 3 min.

The resulting pastes were stuffed into stainless steel cylinder cases (3.1 cm diameter and 3.0 cm height) and wrapped by polyvinylidene chloride film. These pastes were incubated at 30°C for 0, 30, 60 and 120 min (suwari gel) prior to heating at 80°C for 20 min (kamaboko gel) and subsequently cooled immediately in ice water for 5 min. The resulting gels were kept overnight at 5°C prior to the analysis of the gel properties.

Gel strength measurement: After keeping gels at room temperature for 2 h, the gel strength (g cm^{-2}) was estimated by multiplying the breaking strength (g cm^{-2}) and the elongation ($\Delta l/l_0$, Δl breaking length; l_0, sample length) that were measured by stretching test using a rheometer (Model CR-200D; Sun Scientific Co. Ltd, Tokyo, Japan) according to the method of Shimizu *et al.* (1981). For each gel, 6 determinations were performed and the mean value and standard deviation were calculated.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was carried out according to the method of Weber and Osborn (1969), using 3% polyacrylamide gel in a vertical disc-gel system (8.0 cm length, 5 mm diameter).

0.1 g of both surimi sol and kamaboko gel were homogenized using a Teflon homogenizer at 1,200 rpm for 5 min with 4.5 mL of 0.05 M phosphate buffer (pH 6.8) containing 8 M urea, 2% SDS and 0.036 mM N-ethylmaleimide. The obtained homogenate was boiled for 2 min. After boiling, this dissolved solution was cooled in ice water then, kept overnight before preparing unreduced and reduced samples. In order to prepare the reduced sample, 1 mL of the dissolved solution was mixed with 1 ml of the reagent containing 0.05% bromophenol blue in 50% glycerol, 0.4% SDS, 0.5 M phosphate buffer (pH 6.8) and 30% of 2-mercaptoethanol. The unreduced sample was also prepared in the similar manner using the reagent without 2-mercaptoethanol. Ten microliter of the samples was applied to each disc-gel. The protein was stained by Coomassie Brilliant Blue R 250.

Measurement of protein sulfhydryl groups (PSH): The content of protein sulfhydryl groups in surimi sols and kamaboko gels was determined using 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) based on Ellman method (Ellman, 1959). The 0.5 g of surimi sol and kamaboko gel were homogenized by using a Teflon homogenizer at 1,200 rpm for 5 min with 25 mL of 0.1 M phosphate buffer (pH 7.0) containing 8 M urea, 2% SDS, 10 mM ethylene diamine tetraacetic acid (EDTA). Four milliliter of the homogenate was mixed with 0.4 mL of 0.1% DTNB dissolved in 0.1 M phosphate buffer (pH 7.0) containing 8 M urea, 2% SDS and 10 mM EDTA. This reaction mixture was incubated at 40°C for 15 min prior to the absorbance measurement at the wavelength of 412 nm using a Hitachi U-1000 spectrophotometer (Hitachi, Tokyo, Japan). The protein sulfhydryl content was finally calculated using a molar extinction of 13,612 M^{-1} cm^{-1} for 2-nitro-5-triobenzoic acid at this wavelength.

Statistical analysis: Analysis of Variance (ANOVA) was performed and the mean values were compared based on Duncan's multiple range tests (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

First in order to examine the effect of oxidation during setting on the gel formation, the walleye pollack salted surimi was mixed with $KBrO_3$ (0, 2 and 4 μmol g^{-1}) and set at 30°C for 0, 30, 60 and 120 min (suwari gel) prior to heating at 80°C for 20 min (kamaboko gel). Gel properties of these gels were shown in Fig. 1a and b.

In the case of suwari gel at the concentration of 2 and 4 μmol g^{-1} $KBrO_3$, the breaking strength of gels increased more than that of the control gel during setting ($p<0.05$). The elongation also increased dramatically during the first 30 min more than that of the control gel and subsequently decreased to lower values than that of the control with the extension of setting time ($p<0.05$). The gel strength that is evaluated by the multiplication of breaking strength and elongation increased noticeably during setting for 2 h and the increase was higher than the control gel (without $KBrO_3$) ($p<0.05$). These results indicate that $KBrO_3$ enhanced the gel formation of walleye pollack surimi during setting at 30°C.

In the case of kamaboko gel at direct heating at 80°C (without setting), the addition of 2 and 4 μmol g^{-1} $KBrO_3$ increased the gel strength by 391.1 and 171.5 g cm^{-2} respectively, comparing to the control gel.

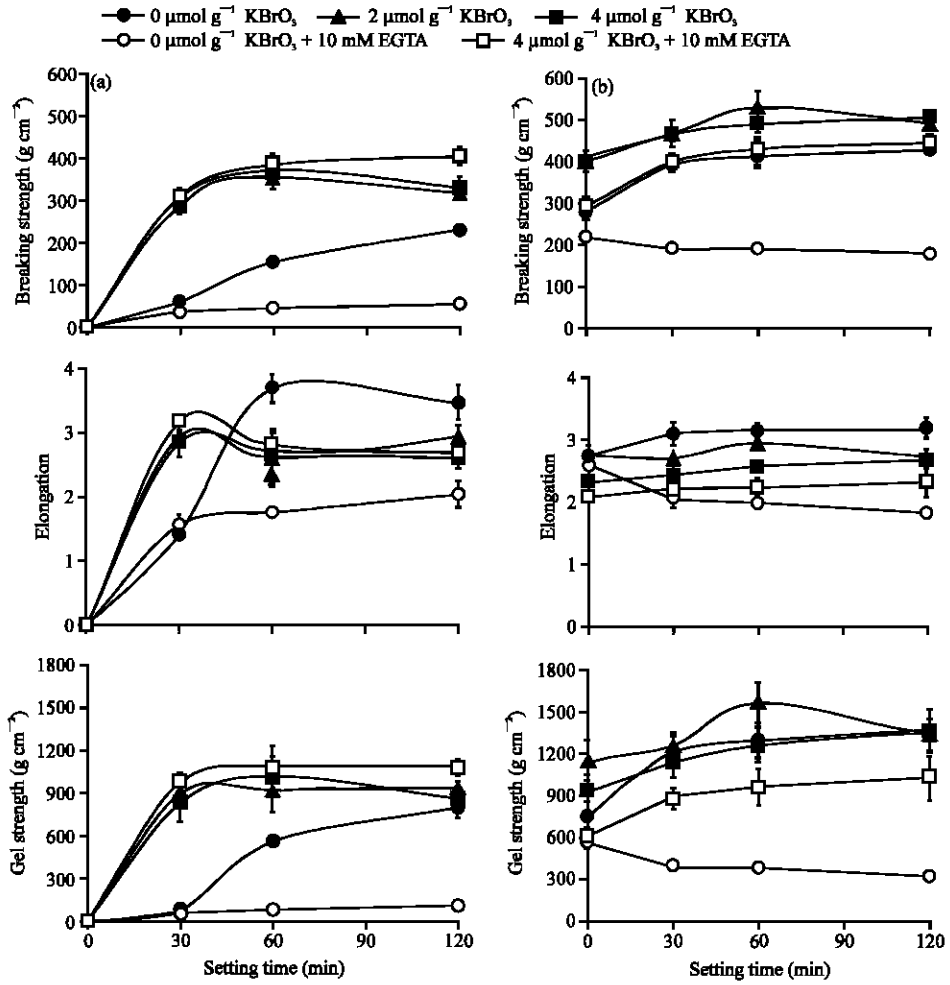

Fig. 1: Effect of $KBrO_3$ on the properties of (a) suwari gel and (b) kamaboko gel of walleye pollack surimi with or without EGTA. Suwari gel was prepared by setting at 30°C for 0, 30, 60 and 120 min and kamaboko gel was prepared by heating at 80°C for 20 min after setting at 30°C for 0, 30, 60 and 120 min

Through setting, the breaking strength of kamaboko gel at both two concentrations of $KBrO_3$ increased higher than the control gel ($p<0.05$). However, the elongation of the gel treated with $KBrO_3$ was lower than that of the control gel, depending on the concentration but regardless of setting time ($p>0.05$). Then the gel strength of kamaboko gel including $KBrO_3$ increased through setting. However, $KBrO_3$ almost did not promote the gel formation of kamaboko gels through setting comparing with the control gel without $KBrO_3$ except the gel treated with 2 μmol g^{-1} of $KBrO_3$ at 60 min setting, where the gel strength was higher than the control gel at the same setting time ($p<0.05$). These results indicate that $KBrO_3$ can enhance gel forming ability of walleye pollack surimi with the appropriate concentration and setting time.

The enhancement of gel formation of walleye pollack surimi during setting is known to relate with the cross-linking between MHC by TGase. Then in order to elucidate the contribution of $KBrO_3$ itself to the enhancement of gel formation during setting, gels were prepared in the presence of 10 mM EGTA to inhibit TGase activity. The gel properties illustrated in Fig. 1 shows that EGTA suppressed the gel formation of suwari gel and kamaboko gel without $KBrO_3$ with the extension of setting time. In the case of suwari gel, the control gel without $KBrO_3$ showed little increase in breaking strength, elongation and gel strength, though $KBrO_3$ added-suwari gel showed the increasing behavior of gel properties even in the presence of EGTA similar to the gel without EGTA.

On the other hand, in the case of kamaboko gel, the values of gel properties of gels without $KBrO_3$ did not increase or slightly decreased in the presence of EGTA through setting ($p<0.05$). In the presence of $KBrO_3$ and EGTA, the breaking strength and the gel strength

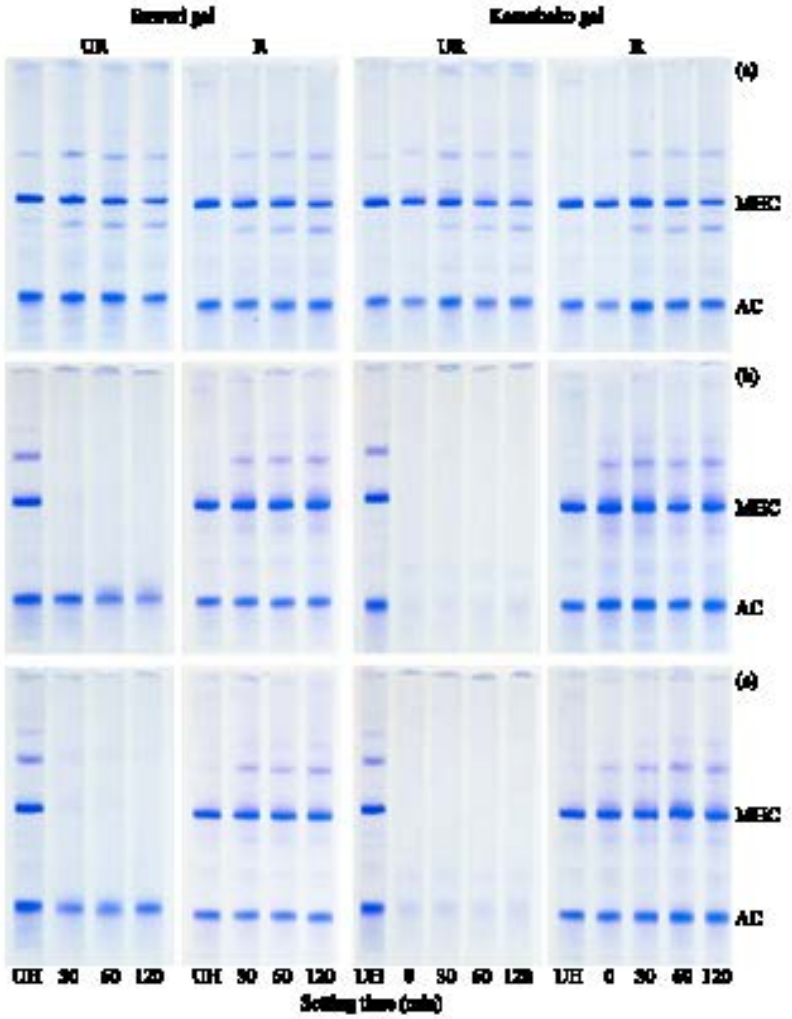

Fig. 2: SDS-PAGE patterns of suwari gels and kamaboko gels of walleye pollack surimi including $KBrO_3$ for (a) 0, (b) 2 and (c) 4 μmol g^{-1}, without EGTA. The gels used are the same as shown in Fig. 1. MHC: myosin heavy chain, AC: actin, UH: unheated surimi sol, R: Reduced samples with 2-mercaptoethanol, UR: Unreduced samples

increased through setting ($p<0.05$), though the elongation did not increase. The breaking strength of the gel with both reagents was lower than that of gel without EGTA and the same as the control gel without both reagents. The elongation was lower than the gels without EGTA and slightly higher than the gel with both reagents. The gel strength was lower than the gels without EGTA but higher than the gel with both reagents. Thus, $KBrO_3$ showed the independent effect on gel formation enhancement through setting. Nevertheless, gel strengthening by the effect of $KBrO_3$ was weaker than ether of the gel treated with $KBrO_3$ (without EGTA) or the control (without both reagents) ($p<0.05$).

In order to examine the behavior of proteins in walleye pollack surimi through setting in the presence of $KBrO_3$ with or without EGTA, the suwari gels and kamaboko gels showed in Fig. 1 were applied to SDS-PAGE analysis. The SDS-PAGE patterns were shown in Fig. 2.

SDS-PAGE patterns of suwari gel without $KBrO_3$ showed that MHC band intensities decreased with the extension of setting time and the higher molecular weight substances (MHC dimer) than MHC appeared above MHC band in both reduced samples and unreduced samples (Fig. 2a). Furthermore, at the same time, the smaller substances than MHC appeared below MHC. The former results indicate that MHC undergoes polymerization via the formation of non-disulfide covalent bonding, which is known as cross-linking catalyzed by the endogenous TGase (Kimura *et al*., 1991; Kumazawa *et al*., 1995). The latter result suggests that the degradation of MHC occurred by protease

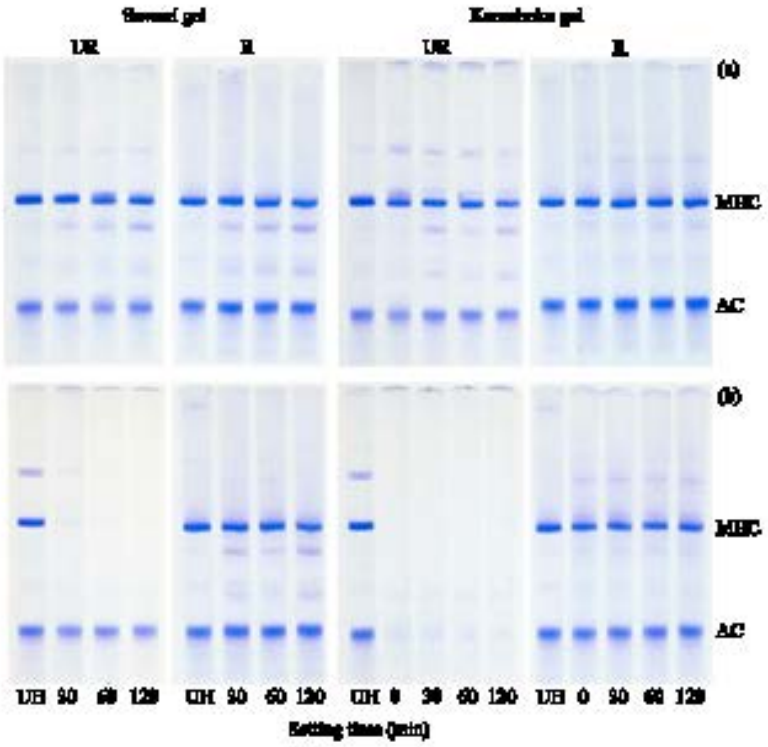

Fig. 3: SDS-PAGE patterns of suwari gels and kamaboko gels of walleye pollack surimi including (a) 10 mM EGTA and (b) 10 mM EGTA + 4 μmol g^{-1} $KBrO_3$. The gels used are the same as shown in Fig. 1. MHC: myosin heavy chain, AC: actin, UH: unheated surimi sol, R: Reduced samples with 2-mercaptoethanol, UR: Unreduced samples

(Hossain *et al*., 2001). Kamaboko gel without $KBrO_3$ also showed the similar patterns to the suwari gels.

In the case of $KBrO_3$-added suwari gels, the formation of MHC dimer was observed in the unreduced samples of gels without setting. This dimer was disappeared in the reduced samples. After setting, the disappearance of MHC was observed in the unreduced samples. MHC and MHC dimer appeared in the reduced samples of suwari gels. The MHC dimer was a substance that was formed by non-disulfide bond during setting. These results show that MHC and MHC dimer were polymerized by disulfide bonding during setting. In the case of kamaboko gels including $KBrO_3$, not only the MHC but also actin were almost polymerized by disulfide bonding regardless of concentration of $KBrO_3$ and setting time (Fig. 2b, c). These results indicate that $KBrO_3$ polymerized MHC through disulfide bonding during setting and heating process and polymerized actin mainly during heating process.

In the case of EGTA-added gels, as shown in Fig. 3a, MHC dimer was not observed in both unreduced samples and reduced samples of suwari gels without $KBrO_3$ as well as in the reduced samples of kamaboko gels without $KBrO_3$. That is MHC intensity was almost constant during setting. However, MHC dimer and polymer on the top position of disc gels were observed in the unreduced samples of kamaboko gels. These results mean that MHC was not polymerized by non-disulfide bonding during setting but polymerized by disulfide bonding during heating at 80°C.

In the unreduced samples of suwari gel treated with $KBrO_3$ (4 μmol g^{-1}), MHC and MHC dimer were disappeared during setting and MHC was subsequently recovered in the reduced samples as shown in Fig. 3b. These results mean that most MHC was polymerized to high molecule substances by disulfide bonds upon setting at 30°C.

SDS-PAGE patterns of kamaboko gels with EGTA showed that MHC and MHC dimer as well as actin were completely polymerized by disulfide bonding regardless of setting time as can be seen by comparing the unreduced samples with the reduced samples. These results indicate that $KBrO_3$ oxidized both MHC and actin to the high molecular substances by disulfide bonding during heating process.

In order to confirm the formation of disulfide bonds in surimi sols and kamaboko gels in the presence of $KBrO_3$, PSH content was determined and shown in

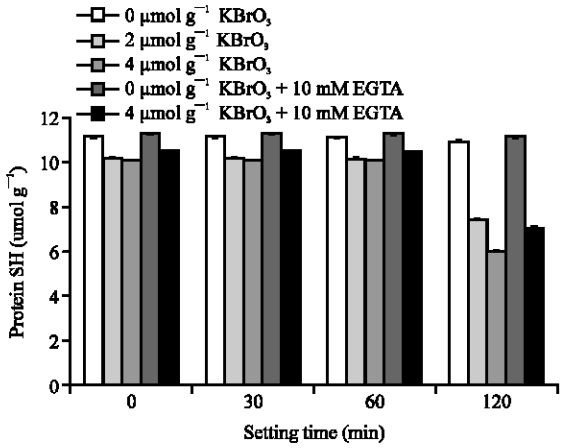

Fig. 4: Content of protein sulfhydryl groups of suwari gels of walleye pollack surimi with or without EGTA. The gels used are the same as shown in Fig. 1

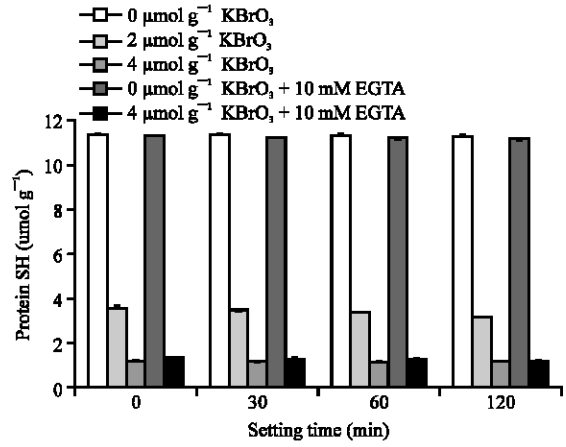

Fig. 5: Content of protein sulfhydryl groups of kamaboko gels of walleye pollack surimi with or without EGTA. The gels used are the same as shown in Fig. 1

Fig. 4 and 5. It has been reported that the decrease in PSH content is a result of the formation of disulfide bonds through the oxidation of sulfhydryl groups (Opstvedt *et al*., 1984).

In the case of suwari gels (Fig. 4), PSH content decreased noticeably corresponding to the increase in the concentration of $KBrO_3$ and setting time ($p<0.05$). That is, PSH content in suwari gel with the addition of $KbrO_3$ of 2 and 4 μmol g^{-1} was less than that in the control gel before setting without $KBrO_3$ by 0.9 and 1.0 μmol g^{-1}, respectively. During setting, PSH content in $KBrO_3$ added-gels decreased by 3.7 and 5.1 μmol g^{-1}, respectively, though that in the control gels without $KBrO_3$ almost did not decrease.

In the case of kamaboko gels (Fig. 5), PSH content decreased further upon heating in the presence of $KBrO_3$ of 2 and 4 μmol g^{-1} by 7.8 to 8.1 and 10.2 to 10.3 μmol g^{-1}, respectively. However, the control gel without $KBrO_3$ did not show the clear decrease in PSH content upon heating ($p>0.05$). These results revealed that $KBrO_3$ oxidized PSH of walleye pollack surimi to disulfide bonds depending on the concentration and setting time. Furthermore, the oxidation of PSH strongly occurred upon heating at 80°C depending on the concentration but regardless of the setting time. Opstvedt *et al*. (2005) reported that the oxidative effects of $KBrO_3$ markedly increase corresponding to the rising of temperature. Runglerdkriangkrai *et al*. (1999) reported that at high temperature, proteins were unfolded and the intra-sulfhydryl groups were exposed to the surface of protein molecules, which coincides with the progress of formation of polymer by disulfide bonds during heating process. Fujita and Itoh (1984) reported that free amino acid cysteine is oxidized to cystine and cysteic acid in the presence of $KBrO_3$ at 80°C.

In the presence of 10 mM of EGTA, PSH content in suwari gels (Fig. 4) and kamaboko gels (Fig. 5) decreased with extended setting time and exhibited the same behavior as that of gel without EGTA, though the decrease in PSH content was slightly lower than that of gel without EGTA. These results show that the oxidation of PSH occurred under the inhibitory condition of TGase similarly to the case without the inhibitor. However, EGTA might suppress the oxidation of proteins owing to the suppressing of unfolding of proteins by chelating calcium ion that induces the unfolding of proteins (Yongsawatdigul and Sinsuwan, 2007).

In the case of suwari gel, gel formation in the absence of $KBrO_3$ was suppressed by EGTA (Fig. 1). This result indicates that EGTA suppressed the formation of MHC dimer through non-disulfide cross-linking by TGase, because MHC dimer was not observed in the reduced samples of EGTA-added gels (Fig. 4). The suppression of gel formation by EGTA is due to the inhibition of TGase activity by chelating calcium ions that is essential to the enzyme (Hossain *et al*., 2001; Wan *et al*., 1994). On the other hand, the gel formation in the presence of $KBrO_3$ during setting was not suppressed with EGTA, though the formation of MHC dimer by non-disulfide bonding was suppressed in the presence of EGTA. In spite of the presence of EGTA, similar extent of oxidation of PSH occurred in the both gels. These results suggest that the gel formation during setting in the absence of $KBrO_3$ is promoted by TGase; an endogenous TGase is known to catalyze the polymerization of MHC via the formation of non-disulfide covalent cross-links during setting (Kimura *et al*., 1991; Kumazawa *et al*., 1995). But that in

the presence of $KBrO_3$ is promoted mainly by the intermolecular oxidation of PSH. However, the formation of MHC dimer by non-disulfide bonding during setting in the presence of $KBrO_3$ (Fig. 2b, c) suggests that TGase in surimi worked some extent even in the presence of $KBrO_3$.

In kamaboko gel, proceeding of gel formation through setting in the presence of $KBrO_3$ was suppressed by EGTA some extent regardless of setting time. This difference can be considered to be the contribution of TGase through cross-linking. In addition, the proceeding of gel formation seems to be related with the extent of oxidation of PSH during mixing and setting, not with the extent of the oxidation upon heating. In other words, the oxidation during mixing and setting might be more contributable to the gel formation than the oxidation upon heating at 80°C. The temperature and the processing stage where the disulfide bonds are formed in surimi might have never been discussed.

In the case of gel formation of control kamaboko gel without $KBrO_3$, the decrease in gel strength through setting was observed in the presence of EGTA. This decrease may be due to the degradation of MHC by protease, because the production of substances between MHC and actin on SDS-PAGE patterns was observed (Fig. 2). The proteolysis observed in this research may be not by Ca-dependent protease, because EGTA chelates only calcium ion. This degradation was slightly suppressed in the presence of $KBrO_3$, as can be seen in the reduced samples of suwari gels and kamaboko gels in Fig. 2 and 3. This result suggests that the cysteine protease was inhibited by $KBrO_3$. Walleye pollack surimi is known to include cysteine protease and serine protease (Hu *et al*., 2008). The inhibition of degradation might contribute to increase gel strength a little, because the decrease in gel strength of gel without $KBrO_3$ by degradation was not so large.

Now we are under confirming the effect of $KBrO_3$ on TGase activity in surimi. Furthermore, we are examining which portion of sulfhydryl groups in MHC is oxidized to form MHC dimer or polymer in order to make clear the difference of the oxidation mechanism from the oxidation in the fish meat without salt.

CONCLUSIONS

In conclusion, it was found that the oxidation of salted surimi sol occurred not only upon heating at 80°C but also during setting at 30°C and mixing with salt in the presence of $KBrO_3$ and that the oxidation during setting prior to heating can contribute to the gel formation through the polymerization of MHC by intermolecular disulfide bonding. However, the contribution of oxidation in surimi seems not to be cooperative with that of TGase.

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