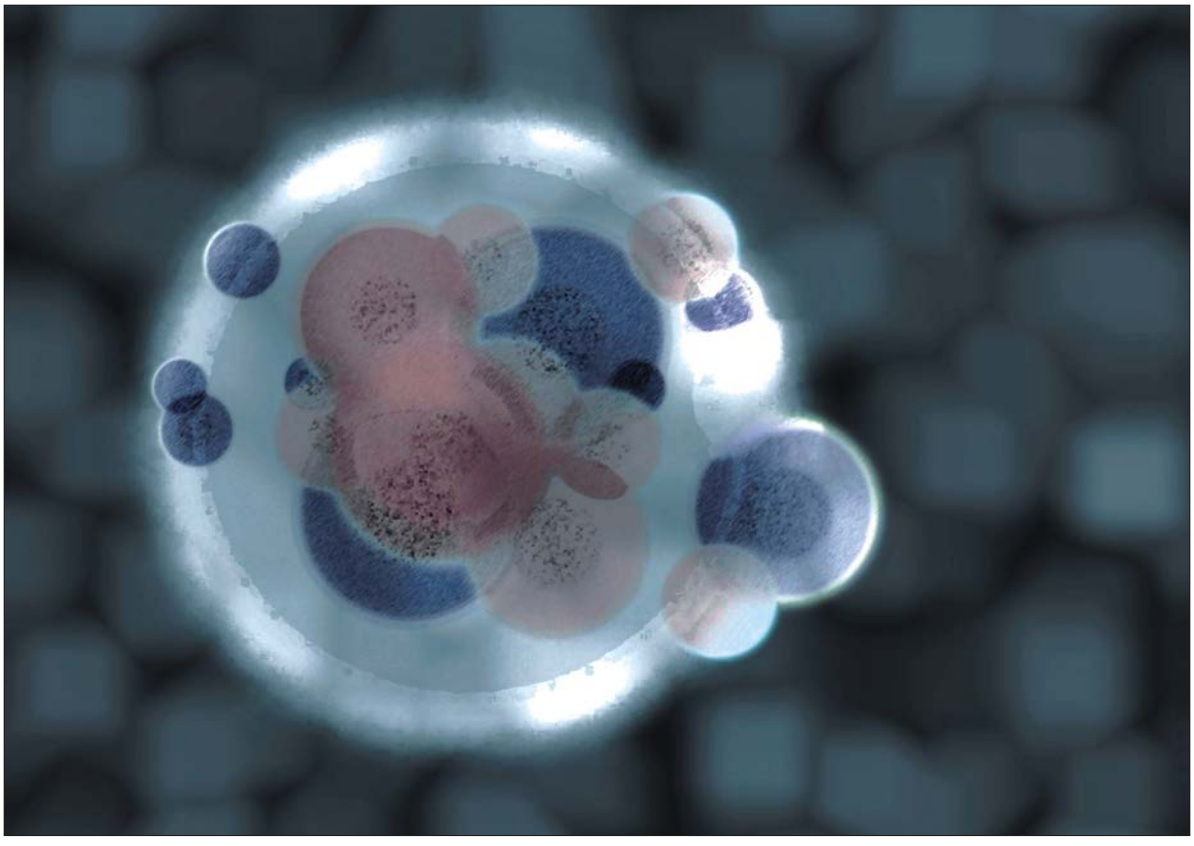

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 10 (1): 18-24, 2010
ISSN 1727-3048
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Effect of Inorganic Oxidizing Reagents on Gel-Forming Properties of Walleye Pollack Surimi through Low Temperature Setting

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Abstract: In order to clarify the effect of oxidation of salted-surimi during setting at low temperature on the gel formation, inorganic oxidants including sodium hypochlorite (NaClO), hydrogen peroxide (H_2O_2) and potassium bromate ($KBrO_3$) were added to walleye pollack surimi with 3% salt and the resulting surimi sols were heated at 80°C for 20 min after setting at 5°C to prepare kamaboko gels. NaClO and H_2O_2 did not act as oxidants and did not affect the gel formation. $KBrO_3$ promoted the oxidation of Protein Sulfhydryl Groups (PSH) already in surimi with the increase in $KBrO_3$ concentration and the prolonging of setting time, accompanying the polymerization of Myosin Heavy Chain (MHC) by disulfide bonding. While the gel formation at direct heating was promoted by $KBrO_3$, the setting did not serve the further increase in gel strength, of which values were lower than that of the gels set without $KBrO_3$. However, under the inhibitory condition of TGase, $KBrO_3$ promoted the gel-formation along with the oxidation of surimi sol during setting. Therefore, the oxidation at the stage of salted surimi sol was considered to contribute to the gel formation, but at the same time decrease the suwari effect by TGase.

Key words: Disulfide bond, gel forming ability, oxidants, setting, surimi

INTRODUCTION

The gel physical property of fish surimi products like kamaboko is one of the important qualities. Oxidizing reagents are known to improve the strength of gels from fish proteins which especially had poor gelling ability, suggesting that the oxidation of sulfhydryl groups to intermolecular disulfide bonds during the process promotes the gel formation of surimi (Kishi *et al*., 1995; Lee *et al*., 1997; Phatcharat *et al*., 2006). However, those gels were prepared by heating at 80°C without preheating or setting process. (Hossain *et al*., 2001a)

On the other hand, setting procedure is now popular in surimi product processing to increase the gel strength. Surimi sols are generally subjected to the temperature in the range from 0 to 40°C (setting) prior to heating at the higher temperatures like at 80-90°C (Alvarez and Tejada, 1997; Kamath *et al*., 1992; Wu *et al*., 1985). During setting, the polymerization of Myosin Heavy Chain (MHC) occurs through cross-linking or isopeptide bond catalyzed by endogenous Transglutaminase (TGase) and this polymerization imparts the enhancement of gel strength after heating at higher temperatures (Benjakul and Visessanguan, 2003; Gilleland *et al*., 1997). In addition, TGase is known as a SH enzyme. In the case of surimi of walleye pollack, a fish species from cold-water region and a popular material for surimi products, setting occurs at low temperature in association with pre-heating process (Kamath *et al*., 1992; Kitakami *et al*., 2004). However, the effect of oxidation of surimi protein during setting at low temperature on the gel formation has not been extensively studied. It is not certain whether oxidizing reagents contribute to the increase in gel formation synergetically or competitively with the TGase.

Therefore, the objective of this study is to investigate the effect of inorganic oxidants on the gel forming properties of walleye pollack surimi during setting at 5°C as well as the polymerization behavior of proteins by SDS-PAGE analysis and the oxidation of sulfhydryl groups.

MATERIALS AND METHODS

Surimi: Walleye pollack (*Theragra chalcogramma*) frozen surimi (SS grade, Maruha- Nichiro Co. Ltd. Japan) was used as a raw material. Moisture and protein contents were measured to be 76.6 and 14.2%, respectively.

Chemical reagents: Inorganic oxidizing reagents, including sodium hypochlorite (NaClO), hydrogen

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peroxide (H_2O_2) and potassium bromate ($KBrO_3$), were obtained from Wako Pure Chemical Industries Co. Ltd, (Osaka, Japan). Ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) was obtained from Dojindo (Kumamoto, Japan).

Chemical composition analysis: The moisture content was determined using infrared moisture determination balance (FD-600-2; Kett Electric Laboratory, Tokyo, Japan). The protein content was estimated by multiplying the nitrogen factor of 6.25 with the nitrogen amount determined by micro-Kjeldahl method (Anonymous, 1998).

Gel preparation: Walleye pollack frozen surimi thawed overnight at 5°C for thawing and was chopped for 1 min using chopper (MK-K48 Matsushita Co Ltd, Japan). The chopped surimi was mixed with the mixture of chilled water (to adjust 80% in moisture), NaCl (3% against 80% moisture surimi) and oxidants (0, 2 and 4 μmol g^{-1} surimi) 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 prepared pastes were set at 5°C for 0, 24 and 48 h (surimi sol), 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 gel strength measurement.

Gel strength measurement: After keeping the gels at room temperature for 2 h, the breaking strength (g cm^{-2}) and the elongation ($\Delta l/l_0$; Δl, breaking length; l_0, sample length) of them 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) and the gel strength (g cm^{-2}) were estimated by multiplying the breaking strength and the elongation. For each treatment, 6 determinations were performed and their mean values 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). The test samples (0.1 g) from each 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 (NEM). The homogenate was boiled for 2 min and the dissolved solution was subsequently cooled in ice water and kept overnight before preparing the reduced and unreduced samples.

In order to prepare the reduced sample, 1 mL of the dissolved solution was mixed with 1 mL of reagent that contains 0.05% bromophenol blue in 50% glycerol, 0.4% SDS, 0.5 M phosphate buffer (pH 6.8) and 20% of 2-mercaptoethanol (a disulfide bond breaking agent). The unreduced sample was also prepared as the preparation of reduced sample with the exclusion of 2-mercaptoethanol. Ten μl of the mixture was applied to each disc-gel. The protein was stained by Coomassie Brilliant Blue R 250.

Measurement of Protein Sulfhydryl Groups (PSH): Total amount of protein sulfhydryl groups in surimi sols and kamaboko gels was determined based on Ellman method (1959) using 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The 0.5 g of both 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 dissolved solution 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 measuring the absorbance 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, 612.5 M^{-1} cm^{-1} for 2-nitro-5-triobenzoic acid at this wavelength.

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

RESULTS

Effect of oxidants on gel forming ability: Effect of various oxidizing reagents on the gel forming ability of walleye pollack surimi was examined at various setting time and the results are shown in Fig. 1a-c. In the case of adding NaClO and H_2O_2, the gel strength as well as the breaking force and the elongation of gels directly heated at 80°C for 20 min, did not increase even with the increase in the oxidant concentrations. The setting at 5°C for 24 and 48 h induced the dramatical increase in breaking force and also gel strength of both gels treated with NaClO and H_2O_2 to similar levels as the controls. NaClO and 2 μmol g^{-1} of H_2O_2 did not affect the enhancement of the gel forming ability ($p>0.05$). In addition, 4 μmol g^{-1} of H_2O_2 showed the less increase than that of 2 μmol g^{-1} of H_2O_2. On the other hand, by adding of $KBrO_3$, the gel strength of no setting gel markedly increased comparing to the control ($p<0.05$). However, by setting, the breaking force

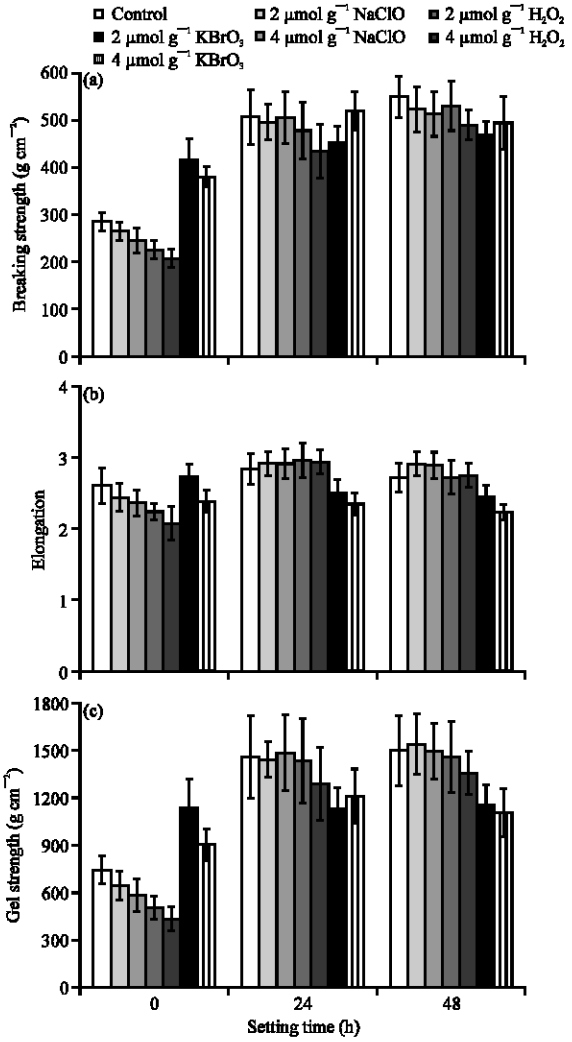

Fig. 1: (a-c) Effect of oxidants on the properties of gels through setting at 5°C for 0, 24 and 48 h prior to heating at 80°C for 20 min

increased slightly and the elongation decreased slightly, resulting in the gel strength seemed not to be affected by $KBrO_3$ and setting time.

In order to examine the protein behaviors by disulfide bonds in walleye pollack surimi through setting in the presence of oxidizing reagents, the salted pastes (surimi sols) and kamaboko gels shown in Fig. 1 were applied to SDS-PAGE analysis, in which the Reduced sample (R) and the Unreduced sample (UR) with 10% of 2-mercaptoethanol was used. The results were shown in Fig. 2. In the case of NaClO and H_2O_2, the decrease in MHC and the increase in MHC dimer were observed in unreduced samples as well as in reduced samples correspondingly to the extension of setting times at each concentration of the oxidants. However, it was observed that MHC and actin band intensities of unreduced samples of surimi sol and kamaboko gel did not change with increasing of oxidant concentration at each setting time as well as those of reduced samples. These results indicate that the polymerization of MHC was not promoted through disulfide bonding by NaClO and H_2O_2. In the presence of $KBrO_3$, the MHC band intensity of the unreduced samples of surimi sol decreased with the increase in the concentration of $KBrO_3$ and with setting time. The MHC and actin band of the unreduced samples of kamaboko gel were completely disappeared regardless of setting time. However, the MHC and actin of the surimi sols and kamaboko gels were recovered after reducing as can be seen in their reduced samples. These results indicate that $KBrO_3$ polymerized MHC through disulfide bonding during all mixing, setting and heating processes. However, since, the decrease of MHC and MHC dimer were also observed through the extension of setting time, $KBrO_3$ seems to inhibit the polymerization of MHC by non-disulfide bonding.

In order to examine the formation of disulfide bonds in surimi sol and kamaboko gel in the presence of oxidizing reagents, the protein sulfhydryl groups were determined and depicted in Fig. 3a and b. In the case of adding NaClO and H_2O_2, it was seen that protein sulfhydryl group content in the surimi sols and kamaboko gels did not change during setting and with the oxidants concentration ($p>0.05$). These results indicate that the disulfide bonds formation by oxidation of sulfhydryl groups did not occur in the presence of NaClO and H_2O_2 through every process of kamaboko gel preparation, or during mixing, setting and heating. In the case of adding $KBrO_3$, the amount of PSH decreased noticeably corresponding to the increase in the concentration of $KBrO_3$ and setting time ($p<0.05$). At the three conditions of setting time 0, 2 and 48 h, the amount of PSH in surimi sol mixed with 2 μmol g^{-1} of $KBrO_3$ decreased by 10, 23 and 29% and that in surimi sol mixed with 4 μmol g^{-1} of $KBrO_3$, 10, 40 and 41%, respectively. In addition, PSH in kamaboko gel at 2 and 4 μmol g^{-1} of $KBrO_3$ decreased dramatically compared with controls and reached almost the same content 62 and 82 % regardless of setting time, respectively.

Effect of $KBrO_3$ on the gel formation in the presence of TGase inhibitor: 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 the oxidative effects of $KBrO_3$ itself to the enhancement of gel formation during

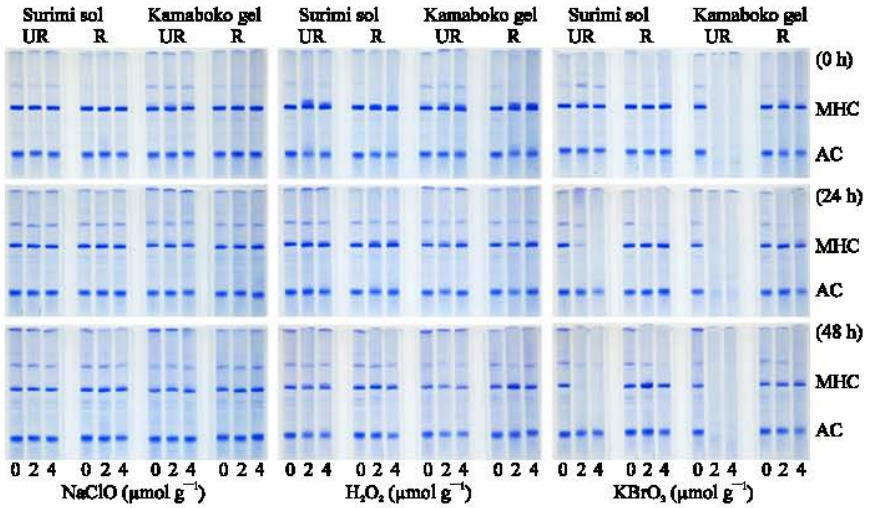

Fig. 2: SDS-PAGE patterns of walleye pollack surimi with oxidants set at 5°C for 0, 24 and 48 h (surimi sol) prior to heating at 80°C for 20 min (kamaboko gel) that are shown in Fig. 1. MHC: Myosin heavy chain, AC: Actin, R: Reduced samples, UR: Unreduced samples

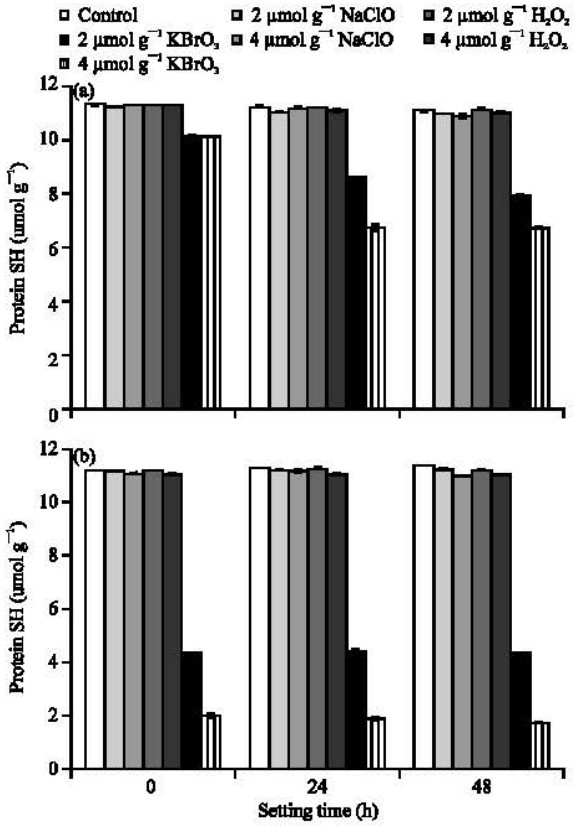

Fig. 3: (a, b) Content of protein sulfhydryl groups of walleye pollack surimi with oxidants set at (a) 5°C for 0, 24 and 48 h (surimi sol) prior to heating at, (b) 80°C for 20 min (kamaboko gel) that are shown in Fig. 1a-c

setting, gels were prepared in the presence of 10 mM EGTA to inhibit TGase activity. Figure 4a-c show that EGTA suppressed the increase in gel strength of the control gel without the oxidant during setting. In the presence of both $KBrO_3$ (4 μmol g^{-1}) and EGTA (10 mM), the increase in gel strength was observed with setting time. Nevertheless, the gel strength was weaker than that of the gel treated with $KBrO_3$ without EGTA and also that of the control gel without $KBrO_3$ and EGTA ($p<0.05$). These results revealed that $KBrO_3$ inhibits the contribution of TGase to the gel formation during setting, though $KBrO_3$ can contribute to the gel formation without setting and through setting.

These sols and gels were applied for SDS-PAGE analysis to differentiate the polymerization caused by disulfide bonding and cross-linking by TGase. The results were shown in Fig. 5a and b. In the unreduced samples of surimi sol treated with $KBrO_3$, MHC and MHC dimer dramatically decreased corresponding to the extended setting time and subsequently MHC was recovered in the reduced samples as shown in Fig. 5b. This result indicates that most of MHC was polymerized to polymer by disulfide bonds even during low temperature setting.

Furthermore, the sols and gels were applied to sulfhydryl determination to confirm the oxidation of protein. The results are shown in Fig. 6a and b. The PSH in surimi sol and kamaboko gel including $KBrO_3$ and EGTA decreased with the extended setting time, ($p<0.05$) and exhibited the same behavior as those of samples without EGTA as shown in Fig. 3a and b.

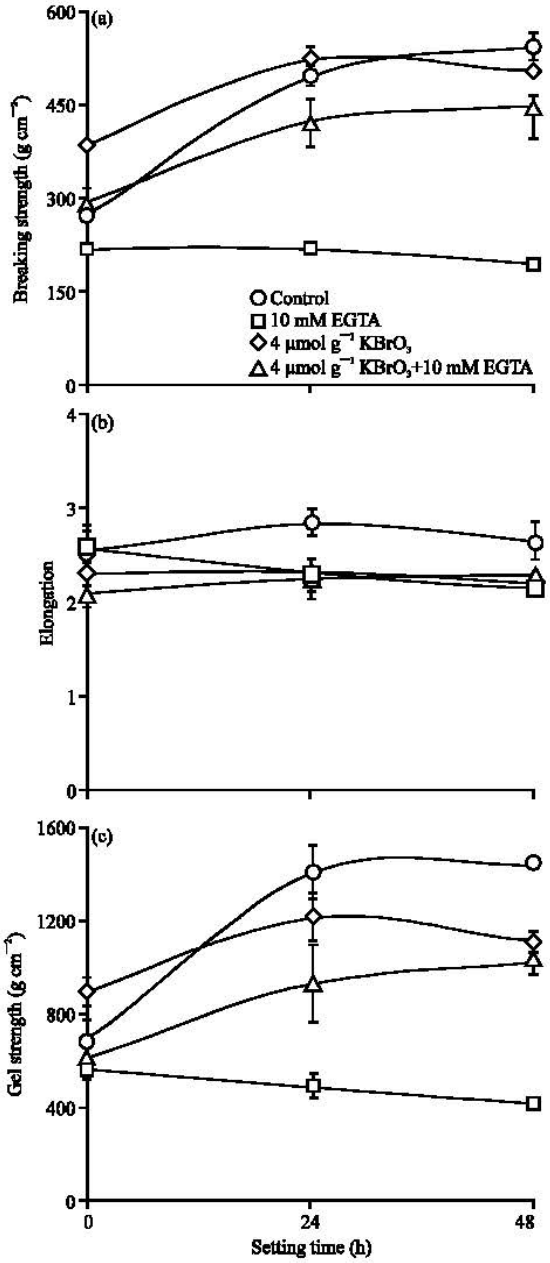

Fig. 4: (a-c)Effect of $KBrO_3$ on the properties of gels through setting at 5°C for 0, 24 and 48 h prior to heating at 80°C for 20 min in the presence of 10 mM EGTA

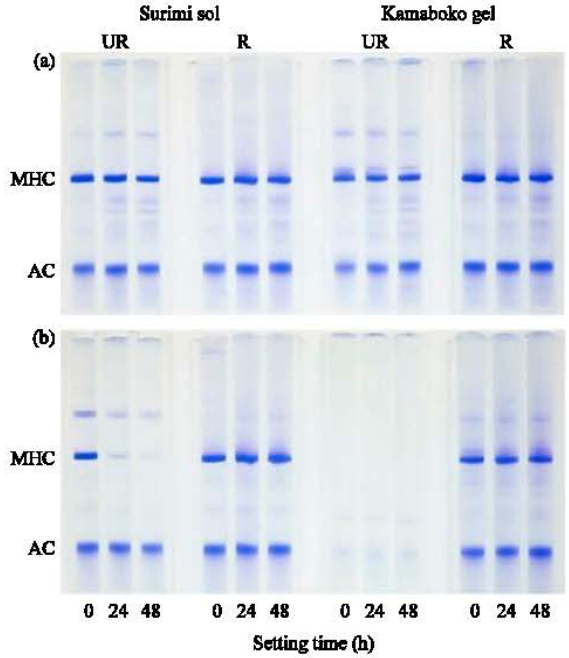

Fig. 5: (a, b) SDS-PAGE patterns of walleye pollack surimi with 10 mM EGTA (a) or 4 μmol g^{-1} $KBrO_3$ and 10 mM EGTA (b) that was set at 5°C for 0, 24 and 48 h (surimi sol) prior to heating at 80°C for 20 min (kamaboko gel). MHC: Myosin heavy chain, AC: Actin, R: Reduced samples UR: Unreduced samples

DISCUSSION

In the case of adding NaClO and H_2O_2, the gel strength of gel without setting did not increase in spite of the increase in the oxidant concentrations. PSH in kamaboko gel as well as in surimi sol was not oxidized even in the presence of NaClO and H_2O_2. In addition, on SDS-PAGE patterns of reduced samples of surimi sols as well as kamaboko gels including NaClO and H_2O_2 at each setting time (Fig. 2) were almost similar to the unreduced samples. This indicates that the polymerization by disulfide bond was not formed by NaClO and H_2O_2.

The increase in gel strength of kamaboko gels including NaClO and H_2O_2 through setting (Fig. 1) is not due to the disulfide bonding of proteins, but is due to the cross-linking by TGase. The formation of polymer by disulfide bonds was not observed (Fig. 2) and the oxidation of sulfhydryl groups did not occur (Fig. 3), moreover MHC dimer and polymer formation was observed in the reduced samples (Fig. 2). This result suggests that therefore, NaClO and H_2O_2 did not act as oxidants in this surimi. The surimi used might not contain metal catalyzer that promotes the oxidation by NaClO and H_2O_2. On the other hand, 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).

In the case of $KBrO_3$ addition, the increase in breaking force and the decrease in elongation of kamaboko gel seems to be related to the oxidation of

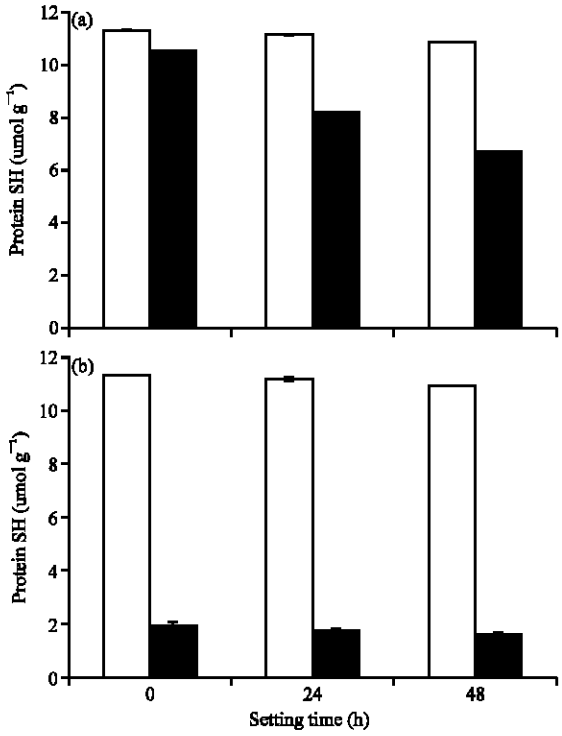

Fig. 6: (a, b) Content of protein sulfhydryl groups of walleye pollack surimi with 10 mM EGTA (□) or 4 μmol g^{-1} $KBrO_3$ and 10 mM EGTA (■) that was set at (a) 5°C for 0, 24 and 48 h (surimi sol) prior to heating at (b) 80°C for 20 min (kamaboko gel)

sulfhydryl groups of surimi sol during setting at low temperature. The oxidation of salted surimi without setting at low temperature did not weaken the gel forming ability but made gels harder in texture. However, the oxidation of fish meat without salt has been reported to weaken the gel forming ability by Tunhun *et al.* (2002). This contradiction might be due to the difference in the position of the oxidized sulfhydryl groups of fish meat or MHC before and after salt grinding or with and without salt.

The increase in gel strength in the presence of $KBrO_3$ at direct heating accompanied with the oxidation of sulfhydryl groups and the polymerization of MHC by disulfide bonds. However, the gel strength of $KBrO_3$-added gel did not increase during setting, different from control gel. Moreover, it was weaker than that of the control gels set for 24 and 48 h. In addition, the decrease of MHC and MHC dimer in unreduced samples of gels including $KBrO_3$ was also observed through the extension of setting time, $KBrO_3$ seems to inhibit the polymerization of MHC by non-disulfide bonding. These results suggest that the enhancement of gel forming ability by oxidation do not act cooperatively with the enhancement by the cross linking by TGase. TGase is known to be SH enzyme, so the effect of $KBrO_3$ on TGase activity in surimi is now under confirmation.

The suppression of gel formation by adding EGTA to surimi is due to the inhibition of TGase activity by chelating calcium ions that is essential to the enzyme (Hossain *et al.*, 2001b; Wan *et al.*, 1994). Therefore, the enhancement of gel formation achieved by $KBrO_3$ in the presence of EGTA is considered by $KBrO_3$ itself. Furthermore, the enhancement significantly depended on setting time and also was related with the oxidation of PSH during setting, not with that during heating, because PSH contents of kamaboko gel at certain content of $KBrO_3$ reached almost the same values in spite of setting time. In addition, oxidation of PSH contributes to the polymerization of MHC, since MHC disappeared during setting and actin remained without polymerization in the unreduced samples on SDS-PAGE patterns. However, after heating, all proteins disappeared in the unreduced samples and appeared again in the reduced samples.

As shown in the reduced samples of surimi sol treated with $KBrO_3$, the production of MHC dimer by non-disulfide bonds during setting was inhibited by $KBrO_3$ (Fig. 2). In other words, the oxidant inhibited TGase activity that polymerizes MHC through cross-linking. Therefore, the enhancement of gel formation of surimi by $KBrO_3$-adding is not cooperative with the gel strengthening through cross-linking by TGase.

It was found that most of MHC was polymerized to the polymer by disulfide bonds even during low temperature setting of walleye pollack surimi (Fig. 2). This result disagrees with the report of Runglerdkriangkrai *et al.* (1999) using actomyosin from carp, flying fish and rabbit, in which at low temperature MHC dimer, not polymer was formed in the oxidative conditions. This difference might be due to the difference of the stability of myosin or actomyosin. In addition, polymerization of MHC through disulfide bonding could strengthen a gel forming ability of a surimi without setting and also might strengthen a surimi that has very weak TGase activity through setting.

CONCLUSION

The inorganic oxidizing reagents, including NaClO, H_2O_2 and $KBrO_3$ have shown different effects on gel forming properties of walleye pollack surimi. NaClO and H_2O_2, did not act as oxidants in the surimi and did not affect the gel formation. $KBrO_3$ could enhance the gel forming ability through polymerization of MHC by oxidation of sulfhydryl groups especially during mixing

and setting processes rather than heating process. However, $KBrO_3$ weakened the promotion of gel formation through cross-linking by TGase.

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