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Gel forming ability of fish meat oxidized during washing

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ABSTRACT: In order to examine the effect of meat oxidation on the gel forming ability before grinding the meat with salt, fish meat was washed with $CuCl_2$ solution, and the gel strength as well as total sulfhydryl (SH) groups and sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) patterns were analysed. Washing with $CuCl_2$ solution resulted in a decrease in the total SH content of fish meat and the formation of myosin heavy chain (MHC) dimer through disulfide bonding. The plot of logarithmic gel strength versus protein concentration after heating the washed meat at 80°C in the presence of 3% NaCl to form a gel illustrated that the gel forming ability of meats washed with $CuCl_2$ solution was weaker than the control meat. The gel of meat washed with $CuCl_2$ showed the polymerization of MHC and MHC dimers through disulfide bonding much more than the control meat gel, although a small decrease in the SH group content after heating. Further washing with ethylene diamine tetra-acetic acid (EDTA) solution to remove $CuCl_2$ from the $CuCl_2$-washed meat also resulted in similar behavior for MHC polymerization and SH content as the $CuCl_2$-washed meat, and the gel was still weaker than the control gel. It was found that the oxidation of SH groups during washing with $CuCl_2$ solution accompanied by MHC dimer formation in the meat results in the weakening of its gel forming ability.

KEY WORDS: **disulfide bonding, gel forming ability, meat washing, myosin heavy chain.**

INTRODUCTION

We have been studying the contribution of disulfide bonding to the gel forming ability of fish meat and investigating the mechanism for disulfide bond formation upon heating. It has been shown that myosin heavy chains (MHC) are polymerized through disulfide bonding[1–3] between the myosin subfragment-1 (S-1) portions upon heating at temperatures above 40°C.[4,5] Whereas, during ice storage[6] or at temperatures below 30°C[5] the oxidation of sulfhydryl (SH) groups was found to form mainly MHC dimers between myosin rod portions. As mention earlier, the behavior of protein molecules through disulfide bonding has been researched mostly at high ionic strengths such as in 0.6 M NaCl, whereas the disulfide bonding of proteins in meat has been investigated mainly at low ionic strengths and, hence, its effects on gel forming ability have not yet been examined.

At low ionic strengths, the structure of myofibril is known to be different from that at high ionic strengths.[7–12] Therefore, it is supposed that disulfide bond formation through the oxidation of SH groups at low ionic strengths might occur in a different manner (or in a different portion of the proteins) from that in high ionic strengths. Furthermore, the effect of disulfide bond formation on gel strength might also be different. Decker *et al.* have examined the oxidative effect of iron and copper in the presence of ascorbate on the physicochemical properties of turkey myofibrils and reported that a decrease in gel strength was accompanied by the polymerization of MHC and actin by disulfide bonds and by the fragmentation of MHC.[13] After investigating other proteins under the conditions of a radical regenerating system, some other researchers have also reported similar results.[14–20]

However, from a preliminary experiment, we found that washing meat with $CuCl_2$ solution

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has oxidizing abilities at low ionic strengths, and is accompanied by MHC dimer formation and no fragmentation of protein. Hence, the $CuCl_2$-treated meat was used to examine the effect of oxidation at low ionic strengths on gel forming ability. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) patterns and total SH content were measured to confirm whether polymerization occurs through disulfide bonding and the oxidation of SH groups.

MATERIALS AND METHODS

Meat

Dorsal white meat of carp (*Cyprinus carpio*) and flying fish (*Cypselurus hiraii*), rabbit meat, and frozen walleye pollack surimi SS grade (Maruha Co. Ltd, Tokyo, Japan) were used.

Preparation of oxidized meats

The oxidized meats were prepared by washing meat using either of the following two procedures.

Non-removal of $CuCl_2$ (Procedure 1)

Minced meat was rinsed twice with 20 volumes of 0.3% NaCl and centrifuged at 3000 ×*g* for 10 min. In order to promote oxidation, the precipitate was homogenized with 4 volumes of 25 p.p.m. or 50 p.p.m. $CuCl_2 \cdot 2H_2O$ in 0.3% NaCl (pH 7) by using a non-bubbling homogenizer at 2000 r.p.m. for 10 min, followed by centrifugation at 3000 ×*g* for 10 min. Control meat was treated with 0.3% NaCl solution without $CuCl_2$. The precipitates were sieved through a stainless mesh to remove any connective tissue. In the present study, the treated meats are referred to as $CuCl_2$-treated meat (oxidized meat) and the control (non-oxidized) meat.

Removal of $CuCl_2$ (Procedure 2)

After the minced meat was rinsed once with 20 volumes of 0.3% NaCl and was treated by the same method as described above (procedure 1) to promote oxidation, it was rinsed with 0.3% NaCl containing 25 mM ethylene diamine tetra-acetic acid (EDTA) (pH 7) in order to remove the $CuCl_2$ in the oxidized meat and to depress the oxidation process. The meat was then centrifuged at 3000 ×*g* for 10 min before any connective tissue was removed using the same method described earlier.

Preparation of cooked gel

The meat gels were prepared from non-oxidized and oxidized meats. Each meat was diluted to three levels of protein concentration at intervals of 1% and mixed with 2.7% NaCl to a final concentration of 3% (w/w). For meats from procedure 1, enough EDTA solution (pH 7) was added to give a final concentration of 25 mM in order to terminate the oxidation of the meat by chelating copper ions, and the meat was then ground for 20 min. The same concentration of EDTA was also added to the control meat. The preparation of meat pastes was performed at 5°C. The meat pastes of various protein concentrations were stuffed into a stainless steel case (30 mm in height, 30 mm in diameter), wrapped in polyvinylidine chloride sheets, heated at 80°C in a water bath for 20 min, and cooled in ice water. The resulting meat gels were refrigerated overnight at approximately 5°C until their gel properties were evaluated.

Assessment of gel properties

The cooled gels were kept at room temperature (20–22°C) for 2 h. The gel properties of each gel was assessed by a Rheometer (Sun Science, Tokyo, Japan) according to the method of Shimizu *et al.*[21] After slicing the gels 5-mm thick and cutting into rings, the gel strength, expressed as a product of tensile strength S (gw/cm^2), and the breaking elongation e ($\Delta l/l_0$) were determined.

Protein determination

Protein concentration was determined by the Kjeldahl method according to the method described by the Association of Official Analytical Chemists.[22]

SDS-PAGE

The meat pastes and gels (0.2 g) were homogenized with 20 mL of 8 M urea–2% SDS–50 mM phosphate buffer (pH 6.8) containing 0.2 mM *N*-ethylmaleimide (NEM) using a Teflon homogenizer. The homogenate was mixed with 10% volumes of 2-mercaptoethanol to prepare reduced samples. Unreduced samples were prepared by adding deionized water instead of 2-mercaptoethanol. The SDS-PAGE of these samples were carried out according to the method of Weber and Osborn[23] using a 3% polyacrylamide disc gel.

Two-step SDS-PAGE

A representative of the first-step SDS-PAGE of the unreduced samples was stained to localize the protein bands. The other gels were used to cut the targeted band. The resulting piece of gel was dipped in 10% 2-mercaptoethanol solution at 40°C for 60 min to undergo reduction. The reduced cut gel was placed on the top of a new gel column completely without any air bubbles, and Bromophenol blue marker was added. Second-step electrophoresis was carried out according to the same procedure as the first step.

Total sulfhydryl content

The total SH group content of pastes and gels were determined according to the Ellman method[24] using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) with some modification. The sample meats (0.1 g) were homogenized with 20 mL of 8 M urea–2% SDS–10 mM EDTA in 0.1 M phosphate buffer (pH 6.8) using a Teflon homogenizer. A volume of 4 mL of homogenate was mixed with 0.4 mL of 0.1% DTNB solution and incubated at 40°C for 15 min. The absorbance at 412 nm was measured on a Hitachi U-1000 spectrophotometer (Hitachi, Tokyo, Japan). The SH content was calculated from the absorbance using the molar extinction coefficient of 13 600/M per cm for 2-nitro-5-thiobenzoic acid at this wavelength.

RESULTS AND DISCUSSION

Gel forming ability of oxidized meat

The gel forming ability of oxidized meat (in which the oxidation was elevated during washing), was investigated by plotting the logarithm of gel strength against various protein concentrations of meat gels, as shown in Fig. 1.

A linear relationship between logarithm of gel strength and protein concentration was observed in both the control and oxidized meat gels. When compared with the control meat, the washing of carp meat with 25 p.p.m. $CuCl_2{\cdot}2H_2O$ solution reduced the gel forming ability. Furthermore, washing in the higher concentration of 50 p.p.m. of $CuCl_2{\cdot}2H_2O$ resulted in decreasing the gel forming ability further. To determine the effect of washing with $CuCl_2$ on the gel forming ability of other meats, flying fish, rabbit, and commercial frozen

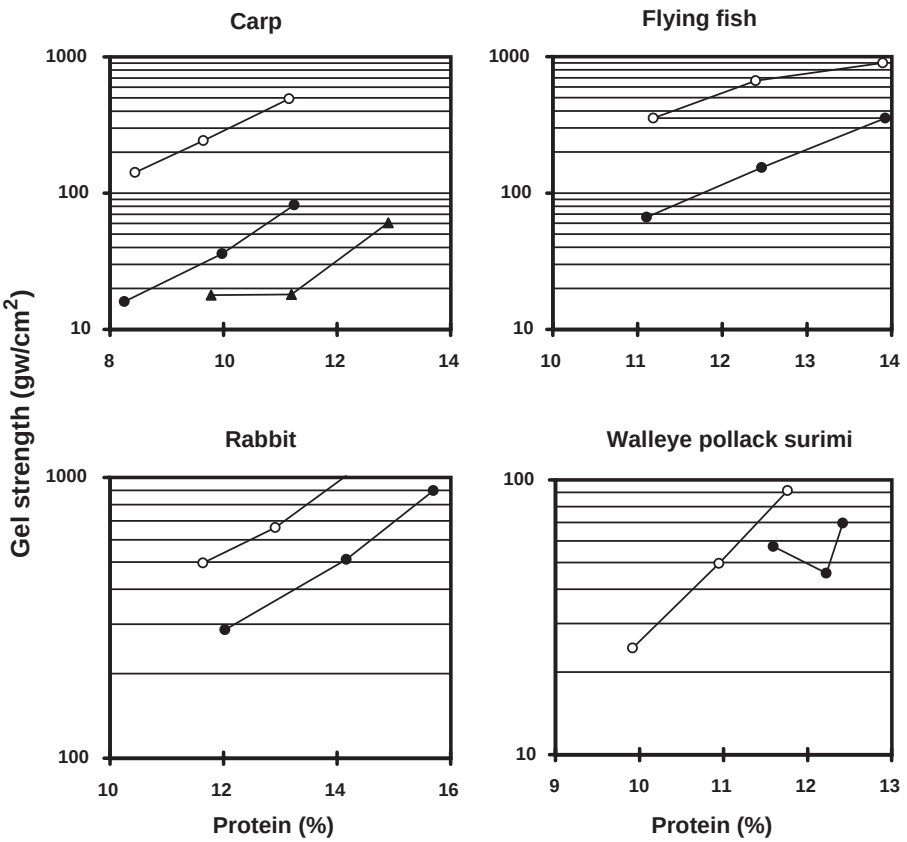

Fig. 1 Gel forming ability of meats prepared by washing with various concentrations of $CuCl_2$ solution: (○) 0 p.p.m. $CuCl_2{\cdot}2H_2O$; (●) 25 p.p.m. $CuCl_2{\cdot}2H_2O$; and (▲) 50 p.p.m. $CuCl_2{\cdot}2H_2O$. Vertical axis is expressed as a logarithmic scale.

walleye pollack surimi were confirmed by treating these meats with the same procedures as carp. In these experiments, 25 p.p.m. $CuCl_2 \cdot 2H_2O$ solution was used as the washing solution because almost all the MHC was oxidized to its dimer form at this concentration.

Each of the meat gels studied demonstrated a linear relationship between logarithm of the gel strength and the protein concentration in a manner similar to the carp gels, except for the $CuCl_2$-treated walleye pollack surimi gel, which did not show a linear relationship. This seems to be due to the the shrinkage of the surimi gel and the release of dripping from the oxidized meat gel, resulting in an increase in protein concentration. A great deal of drip, particularly in the meat gels of lower protein concentration, was assumed to influence the shrinkage of meat gel significantly. Nevertheless, the gel forming ability of oxidized walleye pollack surimi at the altered protein concentration after heating was also lower than that of the control gels.

It is noteworthy that washing the meat with $CuCl_2$ solution decreased the gel forming ability of the washed meat gels.

Changes in sulfhydryl group content by washing and cooking

Figure 2 illustrates the SH group content in the meat pastes and cooked gels. It is obvious that the SH group content in carp meat decreased after washing with $CuCl_2$ solution, but remained unaffected by heating. The decrease in the SH content by washing with 25 p.p.m and 50 p.p.m. $CuCl_2$ solution was 0.84 mole/10^5 g protein and 1.84 mole/10^5 g protein, respectively. Conversely, the decrese in the SH content of these washed meats by heating was 0.44 mole/10^5 g protein and 1.35 mole/10^5 g protein, respectively. The SH content of the control meat decreased to 0.33 mole/10^5 g protein after heating, meaning that the oxidation of SH groups to disulfide bonds occurred mainly during washing with $CuCl_2$ solution. Furthermore, it is suggested that oxidation occurred even in the presence of EDTA for the chelating of copper ions.

In addition, the other three kinds of meat demonstrated a similar decreasing trend in the SH group content as carp. However, the decrease in the SH content of walleye pollack surimi by washing was larger than that of the other meats.

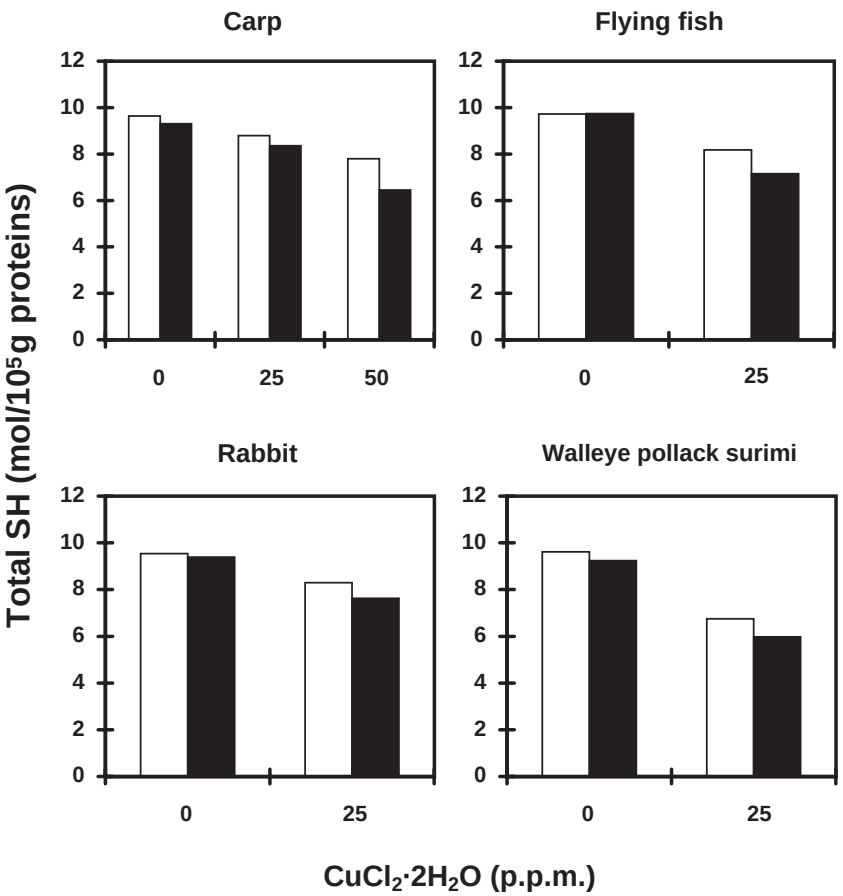

Fig. 2 Total sulfhydryl (SH) content of pastes and gels from the meat washed with various concentrations of $CuCl_2$ solution. (□) Unheated pastes; (■) gels heated at 80°C for 20 min.

Sulfhydryl group content decreased to 2.87 mole/10^5 g protein after washing with $CuCl_2$ solution. Increased disulfide bond formation in the walleye pollack surimi before heating might have affected gel shrinkage.

SDS-PAGE patterns of washed meat and cooked gel

To examine the polymerization behavior of protein in meats washed with $CuCl_2$ solution and heated at various temperatures, SDS-PAGE was carried out.

Unreduced samples of oxidized meat pastes of carp showed a marked decrease in the band intensity of MHC and, concurrently, a new thick band (band B) appeared, which seemed to be double the molecular weight size of MHC (Fig. 3). In the reduced samples, the band B disappeared, suggesting that MHC was oxidized to the MHC dimer by disulfide bonding. Along with the results from the two-step SDS-PAGE analysis of band B, it was clearly demonstrated that band B was composed of the MHC monomer, which is formed through disulfide bonds (Fig. 4a). In addition, the top polymer (band A) on SDS-PAG was found, along with a decrease in the actin band in the unreduced samples. Because this band A was dissociated by 2-mercaptoethanol (Fig. 4b), it was assumed that it was formed from intact myosin molecules and actin through disulfide bonding. Conversely, control meat pastes showed a different SDS-PAGE pattern. No changes in the existing MHC band and no formation of any new band was observed.

After heating, unreduced samples of the control meat gel showed a slight decrease in MHC and actin together with the formation of three new bands; namely, the bands A, B and C, which were referred to as the top polymer, the dimer band, and the band just above the MHC. These three bands

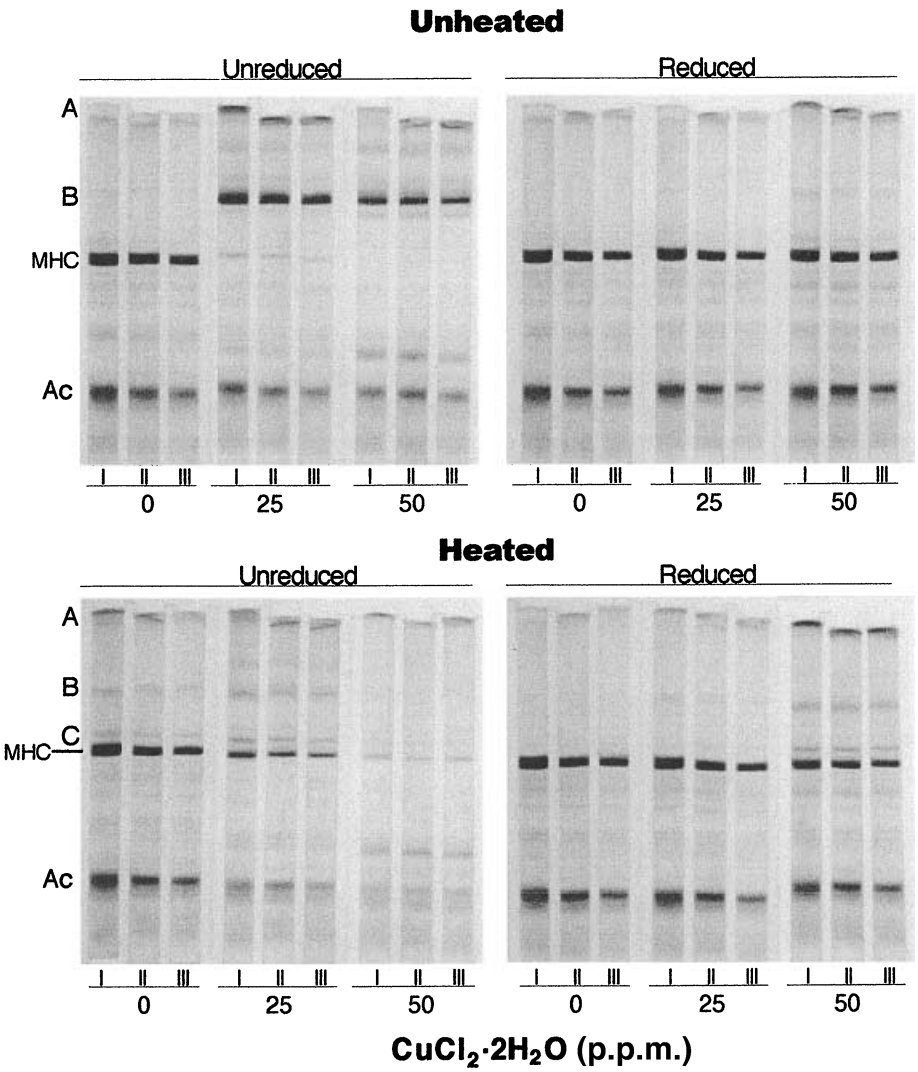

Fig. 3 Sodium dodecylsulfate–polyacrylamide gel electrophoresis patterns of pastes and gels prepared from carp meat oxidized by washing with 0 p.p.m, 25 p.p.m and 50 p.p.m. $CuCl_2 \cdot 2H_2O$ solutions. I, II, and III indicate high, middle and low levels of protein concentration samples, respectively. MHC, myosin heavy chain; Ac, actin; Unreduced and Reduced, samples of pastes or gels solubilized without and with 10% 2-mercaptoethanol, respectively; Unheated, pastes; Heated, gels heated at 80°C for 20 min; A, B and C, unknown bands.

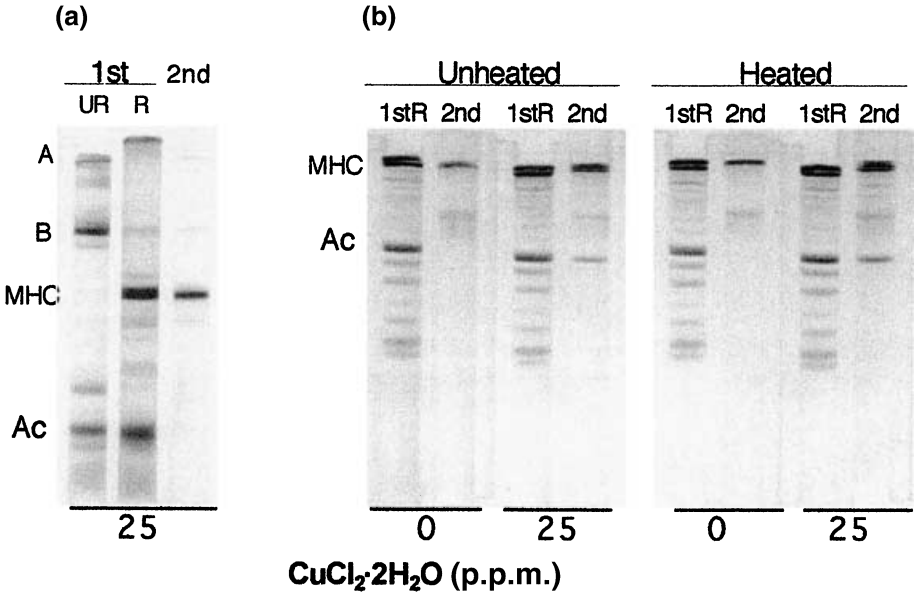

Fig. 4 Two-step sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (2nd) patterns of A and B bands on the first-step SDS-PAGE (1st) patterns of carp meat gels oxidized by washing with 0 p.p.m. and 25 p.p.m. $CuCl_2{\cdot}2H_2O$ solutions. (a) Two-step SDS-PAGE of B band using 3% polyacrylamide gel; (b) two-step SDS-PAGE of A band using 10% polyacrylamide gel; UR and R, samples of pastes or gels solubilized without and with 10% 2-mercaptoethanol, respectively.

disappeared after reduction, indicating that they were formed by disulfide bonding; whereas, unreduced samples of the $CuCl_2$-treated meat gel showed a marked decrease in the MHC dimer along with the disappearance of actin. Concomitantly, band C increased slightly and the formation of polymer (band A), which could not migrate, was observed on the surface of 3% SDS-PAG. This polymer could not be seen on the SDS-PAGE photograph. The polymerization of MHC by disulfide bonding was greater in the oxidizing agent of higher concentration (i.e. 50 p.p.m. $CuCl_2{\cdot}2H_2O$) because in the unreduced samples almost all of the MHC monomer and dimer disappeared, but the MHC monomer reappeared in the reduced samples. The band A of SDS-PAG in unreduced samples was analysed by two-step SDS-PAGE (Fig. 4b), which demonstrated that band A comprised mainly MHC monomer and actin. This band A was formed through disulfide bonding upon heating. The band C was observed on the heated meat gel, whereas it was invisible in the meat paste before heating. According to its mobility and from two-step SDS-PAGE analysis (data not shown), band C seems to be a combined substance of myosin with actin. The formation of MHC monomer in the 25 p.p.m. $CuCl_2{\cdot}2H_2O$-washed sample after heating might be due to the breaking of the MHC dimer to the monomer through further oxidation of the disulfide bonds to SO_3H. However, in the 50 p.p.m. $CuCl_2{\cdot}2H_2O$-washed sample, the MHC monomer was polymerized again by disulfide bonding, which might be due to the oxidation of any remaining SH groups.

The other kinds of meats studied demonstrated an oxidative effect on SDS-PAGE patterns in the same manner as carp. (Fig. 5) In every reduced sample of the various oxidized meat gels, fragmentation of protein was not observed. This is different from the results of a free-radical regenerating system.[14–20] Therefore, the decrease in gel forming ability is not due to the fragmentation of protein.

Effect of $CuCl_2$ removal from oxidized meat on gel forming ability

As shown earlier, the SH content of oxidized meats decreased after heating at 80°C (Fig. 2), and the polymerization of MHC and actin by disulfide bonding occurred; whereas, such behavior was not observed in the control meat (Fig. 3). The question remains whether enough EDTA was added to terminate the oxidation and polymerization of MHC and actin completely as the molar concentration of the 25 p.p.m. $CuCl_2{\cdot}2H_2O$ was 0.15 mM and that of the EDTA was 25 mM.

In order to confirm the effect of any remaining $CuCl_2{\cdot}2H_2O$ on oxidized meat, removing copper ions by rinsing with 25 mM EDTA solution was considered as a further step after washing with $CuCl_2$ solution in the preparation of oxidized meat. The gel strength of meat gels are shown in Fig. 6. The EDTA-washed meat was also weaker in its gel forming ability than the control meat. The amount of SH groups decreased slightly after heating in the EDTA-washed meat (Fig. 7). The SDS-PAGE pattern of EDTA-washed meat also showed MHC dimer

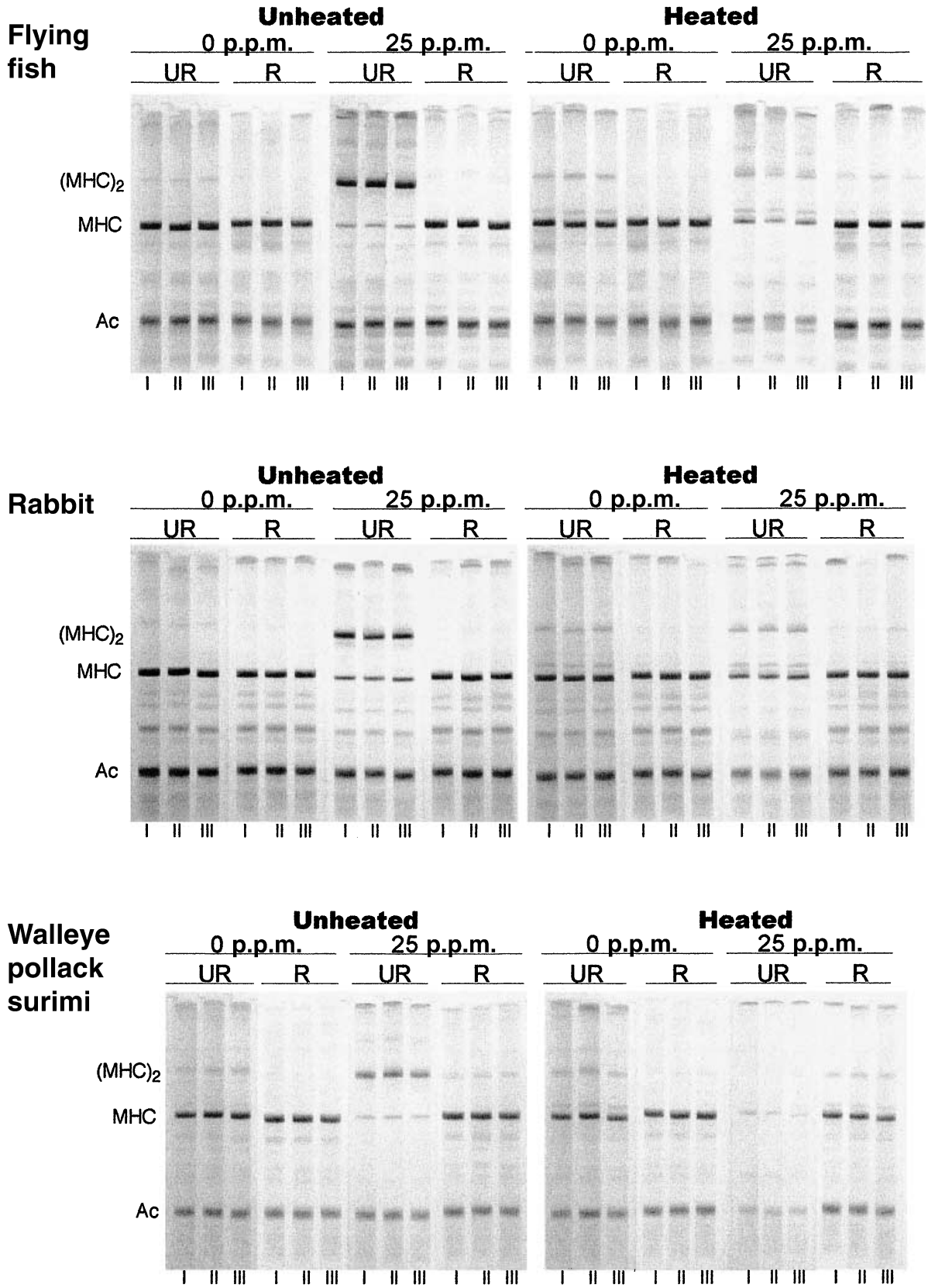

Fig. 5 Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) patterns of pastes and gels prepared from three kinds of oxidized meats of rabbit, flying fish and walleye pollack surimi by washing with 0 p.p.m. and 25 p.p.m. $CuCl_2{\cdot}2H_2O$ solutions. I, II, and III indicate high, middle and low levels of protein concentration, respectively. MHC, myosin heavy chain; $(MHC)_2$, myosin heavy chain dimer; Ac, actin; UR and R, samples of pastes or gels solubilized without and with 10% 2-mercaptoethanol, respectively.

formation through disulfide bonding in the unheated meat paste, and showed MHC polymer formation through disulfide bonding along with a decrease in actin in the heated gels (Fig. 8), which was behavior that was almost similar to that shown in Fig. 3, although the amount of MHC monomer did not increase. These results indicate that any copper ions remaining in the $CuCl_2$-treated meat does not affect the polymerization of MHC and actin upon heating at 80°C. Disulfide interchange reaction is known to be a polymerization reaction of protein that occurs between a disulfide compound and a sulfhydryl compound in the presence

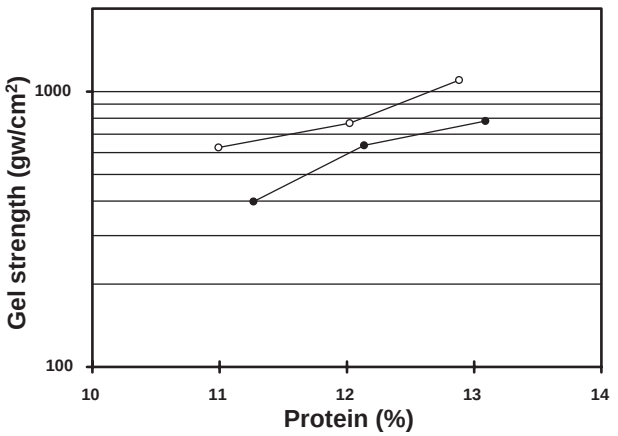

Fig. 6 Gel forming ability of gels prepared from carp meat oxidized by washing with $CuCl_2$ solution and washed once with 25 mM ethylene diamine tetra-acetic acid (EDTA) solution. (○) 0 p.p.m. and (●) 25 p.p.m. $CuCl_2{\cdot}2H_2O$. Vertical axis is expressed as a logarithmic scale.

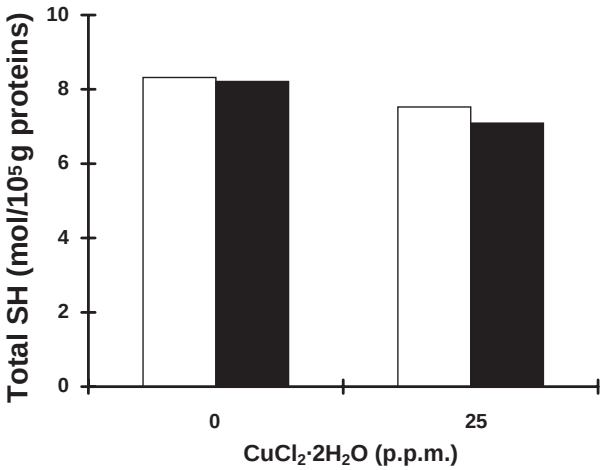

Fig. 7 Total sulfhydryl (SH) content of pastes and gels prepared from carp meat that was washed with 0 p.p.m. and 25 p.p.m. $CuCl_2{\cdot}2H_2O$ solutions and washed once with 25 mM ethylene diamine tetra-acetic acid (EDTA) solution. (□) Unheated pastes; (■) gels heated at 80°C for 20 min.

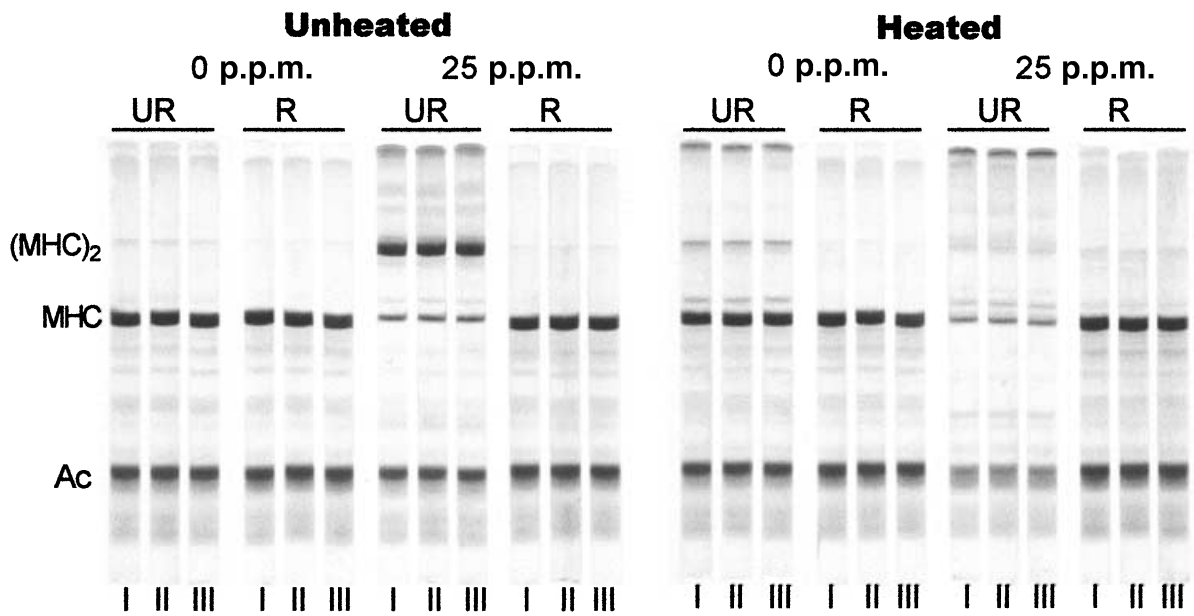

Fig. 8 Sodium dodecylsulfate–polyacrylamide gel electrophoresis patterns of pastes and gels prepared from carp meat that is oxidized by washing with 0 p.p.m. and 25 p.p.m. $CuCl_2{\cdot}2H_2O$ solutions and followed by washing with 25 mM ethylene diamine tetra-acetic acid (EDTA) solution. I, II, and III indicate high, middle and low levels of protein concentration, respectively. MHC, myosin heavy chain; $(MHC)_2$, myosin heavy chain dimer; Ac, actin; UR and R, samples of pastes or gels solubilized without and with 10% 2-mercaptoethanol, respectively.

of EDTA to inhibit oxidation.[25–27] Therefore, the possibility of this reduction should be considered.

The aforementioned results raise another question. Although washing with $CuCl_2 \cdot 2H_2O$ solution promoted MHC dimer formation through the oxidation of SH groups and the heated gel of the oxidized meat showed much greater amounts of polymer formation of MHC and actin compared with the non-oxidized meat, the gel forming ability of the oxidized meat was lower than that of the control meat. It has been reported previously that the formation of disulfide bonds in the presence of high salt concentrations such as 0.6 M NaCl is beneficial to strengthening the gel network during thermal gelation.[7,28,29] However, because the structure of muscle protein at low salt concentrations is different from that at high salt concentrations, it is supposed that the portion of disulfide bonding in muscle protein is also different in solutions of high salt concentrations compared with that of solutions of low salt concentrations. The disulfide bonding in washed meat before heating might hinder the interactions between proteins and, hence, inhibit gel formation.[15]

In conclusion, it should be noted that the oxidation of fish meat through the formation of disulfide bonds during washing with $CuCl_2$ solution is responsible for the decreased gel forming ability of fish meat. Therefore, it is proposed that the formation of disulfide bonds through the oxidation of SH groups in the meat before grinding results in reducing gel forming ability. As the addition of oxidant to salt-ground meat before heating increases gel strength,[29,30] it would be of interest to clarify whether the addition of $CuCl_2 \cdot 2H_2O$ to non-oxidized meat after grinding with salt causes a difference in the oxidative effect on gel forming ability and the polymerization behavior of protein molecules, which will be reported in a future paper.

From the viewpoint of the practical processing of washing meat, tap water for washing contains the oxidant NaClO and is not pure water; hence, oxidation may occur during washing with tap water. The present study's results suggest that it is worth investigating whether or not oxidation occurs during washing.

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