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Main protein components in frozen surimi contributed to heat-induced gel formation

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Abstract

Directly heated gel (90°C) and two-step heated gel (25 and 90°C) were prepared from surimi made from arabesque greenling and walleye pollack, either with or without added 3% albumen powder (AP). The resulting heat-induced gels were solubilized in solvents containing 0.6 M NaCl, 1.5–8.0 M urea, 2% mercaptoethanol, and 2% sodium dodecyl sulfate (SDS) in various combinations, and the compositions of the dissolved proteins were analyzed by SDS-polyacrylamide slab gel electrophoresis. For greenling, the main proteins contributing to the heat-induced gel structure were myosin heavy chain (MHC) and actin (AC) in the case of directly heated gel, and were AC, MHC, and unidentified component X1 (140.2 kDa) in the two-step heated gel. In either gel type, the formation of the gel structure was suggested to involve strong disulfide bonds and hydrophobic interactions. On the other hand, for pollack, the gel structure formation was suggested to involve MHC and AC in the directly heated gel and MHC polymer and AC in two-step heated gel, through the concerted action of strong hydrophobic interactions, disulfide bonds, and isopeptide bonds. However, the addition of 3% AP did not result in any notable changes, regardless of the fish species.

Introduction

Kamaboko, a type of Japanese heated fish paste, is a highly nutritious animal protein food product that has been enjoyed for a long time in Japan. The primary raw ingredient of kamaboko is frozen surimi (minced meat) from walleye pollack *(Theragra chalcogramma)*, which was originally developed to enable the stable long-term preservation (over several years) of the gel-forming ability that is characteristic to fish muscular protein. Accordingly, to create kamaboko products that appeal to the Japanese taste, it is necessary to examine the protein chemistry of the processes related to the gel-forming ability of the key ingredient, surimi. However, the majority of the protein-chemistry studies performed thus far have utilized myosin as their model. Consequently, these studies investigated the gel-forming ability of myosin, and it is questionable whether such results can be applied directly to kamaboko products. For instance, the difference between gel formation that does or does not involve suwari (a specific setting process in which salted and ground fish meat sets to an elastic gel-forming state when left standing at a low to moderate temperature) has yet to be explained from the viewpoint of protein-chemistry. It goes without saying that surimi is composed of multiple proteins rather than a single protein; it is therefore necessary to elucidate the movements of all protein components concurrently in relation to the physical properties of kamaboko being formed.

In our previous report, we attempted to identify the types of chemical bonds between the proteins involved in the formation of gel structures in kamaboko (heat-induced gel) from the variations in solubility and time-dependent changes of the proteins. To do so, we dissolved heat-induced gels in various solvents comprising a mixture of salt, urea, mercaptoethanol, and sodium dodecyl sulfate (SDS) (Kunimoto *et al*., 2014). We have also conducted a similar study taking the effects of additive proteins such as albumen powder (hereafter "albumen"), which are frequently used as texture reinforcements to increase the elasticity of kamaboko, into consideration (Kunimoto *et al*., 2013). The results of these studies indicated that the types of chemical bonds between the proteins involved in gel structure formation are completely different in heat-induced gels with and without the suwari setting process. The proteins in heat-induced gels with suwari were held together by

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strong intermolecular forces, whereas those in heat-induced gel without suwari were held together by relatively weak forces, and this difference influenced the physical property of kamaboko (Kunimoto *et al*., 2014). In addition, the results suggested that the texture-reinforcing effect of albumen involves disulfide bonds. However, no light could be shed on the specific types of intermolecular forces acting between the numerous protein components of surimi, as far as the structural formation of heat-induced gel is concerned.

In this study, we analyzed the composition of proteins that were dissolved in various solvents containing salt, urea, mercaptoethanol, and SDS, using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). By identifying the protein components and types of intermolecular forces involved in the gel structure, we attempted to determine their involvements in the suwari gel-setting process and the reinforcing function performed by albumen (Cheftel *et al.*, 1988).

Materials and Methods

Samples

On-land processed second-grade frozen surimi made from arabesque greenling (*Pleurogrammus azonus*, hereafter simply "greenling") and walleye pollack (Theragra chalcogramma, hereafter simply "pollack") were used as samples. Their moisture and protein contents were 76.7% and 14.2%, respectively, for greenling and 79.0% and 13.8% for pollack. The pH was 7.16 and 7.44 for *P. azonus* and *T. chalcogramma*, respectively. The moisture- and protein contents of the dried and albumen powder (K-type, Kewpie Corporation) added to the heat-induced gels were 6.4% and 82.5% respectively, and its pH was 7.49.

Preparation of heat-induced gels

Frozen surimi stored at -50°C was defrosted on the day preceding the experiment by transferring to a -20°C refrigerator. It was then minced with a high-speed cooling vacuum food processor (Universal Machine UM-5; Stephan Machinery GmbH, Hameln, Germany) and ground with 3% NaCl (w/w). Albumen was added to some samples at 3% (w/w) after the salt-grinding process. Temperature was regulated throughout the grinding process so that the final ground meat paste remained at or below 9°C. Polyvinylidene chloride tubes with folding radius of 48 mm were stuffed with the prepared ground meat paste and preheated for 8 to 9 h in a constant temperature water bath set at 25°C. At set time points, some tubes were removed, heated for 30 minutes at 90°C, and then chilled immediately in iced water. These pastes were defined as two-step heat-induced gel (two-step heated gel). Pastes heated directly for 30 minutes at 90°C without preheating were defined as directly heat-induced gel (directly heated gel).

Measurement of heat-induced gel protein solubility in various solvents

Heat-induced gels were shredded to the appropriate size of minced gel of less than 3 mm in length and width and batches of approximately 0.27 g were weighed out, which were subsequently dissolved in 4 ml of solvents of various compositions by agitation at room temperature for 48 h (Kunimoto *et al*., 2013). The solvents used were S1 (0.6 M NaCl), S2 (0.6 M NaCl + 1.5 M urea), S3 (0.6 M NaCl + 8.0 M urea), S4 (0.6 M NaCl + 8.0 M urea + 2% 2-mercaptoethanol (Me)), and S5 (2% SDS + 8.0 M urea + 2% Me). Protein solubility was measured for each of the five solvent types. Composition of the solvents and presumed types of inter-protein bonds that each disrupts are summarized in Table 1. All solvents were adjusted to pH 7.5 with 20 mM Tris-HCl buffer.

Sample solutions with dissolved proteins were centrifuged (2100×g, 20 min). A fixed quantity was taken from their supernatant and diluted 10 times (v/v) with water, and trichloroacetic acid was added at a final concentration of 7.5% to precipitate the proteins. Precipitates were collected by centrifugation (2100×g, 20 min) and a small quantity of petroleum ether was added, mixed, and centrifuged (2100×g, 20 min) again to remove the petroleum ether with dissolved lipid. The remaining pellets were dried and dissolved in 1.0 M sodium hydroxide to measure protein quantities by the Biuret test, for the final determination of protein solubility in each solvent type. Measurements were performed in triplicate and average values were obtained. The all the data was expressed as mean ± standard deviation.

Composition analysis of heat-induced gel proteins dissolving in various solvents

Next, we attempted to elucidate the main protein components involved in the structural formation of heat-induced gels and the degree of involvements of each protein. For this purpose, we analyzed the composition of proteins dissolving in the aforementioned five types of composite solvents using SDS-PAGE.

Samples for SDS-PAGE were prepared from the supernatant of protein solutions so that the final protein concentrations were at a fixed value. As those

Table 1. Chemical composition of solvents used for solubilization and breakable type of binding forces

Solvents	Composition	Symbols	Breakable type of binding forces
(S1)	0.6 M NaCl, pH 7.5	N	Ionic bond
(S2)	0.6 M NaCl + 1.5 M urea, pH 7.5	N + 1.5 M U	Ionic bond, Hydrogen bond
(S3)	0.6 M NaCl + 8.0 M urea, pH 7.5	N + 8.0 M U	Ionic bond, Hydrogen bond, Hydrophobic interaction
(S4)	0.6 M NaCl + 8.0 M urea + 2% mercaptoethanol, pH7.5	N + 8.0 M U + Me	Ionic bond, Hydrogen bond, Hydrophobic interaction, Disulfide bond
(S5)	2% SDS + 8.0 M urea + 2% mercaptoethanol, pH 7.5	SDS + 8.0 M U + Me	Ionic bond, Hydrogen bond, Hydrophobic interaction, Disulfide bond, Other intense bonds

*pH 7.5: 20 mM Tris-HCl buffer

proteins components that dissolved in S1 are easily soluble in 0.6 M NaCl, they are thought to bind to the heat-induced gel structure using relatively weak forces, compared to those proteins that are eluted only when in the presence of 1.5–8.0 M urea, 2% Me, or SDS. Protein solubility in S1 was 1.5–13.5%, the lowest among all solvents (Figure 1). Such S1-soluble proteins could not permit precise SDS-PAGE analysis. The reason is that the protein concentrations were too small to quantitate the protein components. So, we did not analyze S1 on its own.

SDS-PAGE was performed according to Laemmli's method (Laemmli, 1970), using commercial polyacrylamide slab gels (5.0% and 7.5%, PAGEL NPU-5 L, and 7.5 L, ATTO Corporation). The amount of protein in each sample was set to 10 µg. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R250 solution with 45% methanol and 9% acetic acid, and then destained by 5-15 minute immersions in 45% methanol-9% acetic acid solution to remove excessive pigment. Gel images (Figure 2) were digitized into a computer with a scanner (CanoScan LiDE 700F, Canon; 300 dpi and more) using a red light source. The staining intensity of each band was measured using image analysis software (ImageJ, National Institute of Health, USA), by which determination limit of staining density of each band are to 10 ng. The quantity of each protein component was expressed as a percentage (%) of the total staining intensity (Figure 3). SDS-PAGE electrophoretograms revealed easily identifiable proteins present in large quantities, such as myosin heavy chain (MHC), actin, and tropomyosin, as well as several unidentifiable protein components (hereafter, labeled with "X") in minute quantities.

In this study, we classified the heat-induced gel protein components into nine categories—Xn, MHC, X1, X2, X3, X4, actin, tropomyosin, and X5—in the order of least to most distance migrated during electrophoresis (i.e. largest to smallest molecular weight). In directly heated gels, MHC, actin, and tropomyosin were the main constituents. In two-step heated gels, additional main components were observed, namely X1 in greenling and Xn and X1 in pollack. In contrast, components X2, X3, and X5 were relatively minute in quantity. Component X4 was subdivided into X4a and X4b, as it was comprised in some cases of two components of close molecular weight. Relative mobility and assumed molecular weight of those unidentified components were calculated from their migration distance on electrophoretogram, using a commercial protein size standard (PageRuler unstained protein ladder, Thermo Scientific). The results are summarized in Table 2. All unidentified components were common to both types of heat-induced gels.

Table 2. Relative mobility and assumed molecular weight of soluble protein components in heat-induced gel by SDS-PAGE analysis

Protein components in heated gel (Symbol)	Relative mobility on 7.5% acrylamide gel rod	Approximate molecular weight (kDa)
Xn	—	—
MHC	1	215.7
X1	1.42	140.2
X2	1.74	109.6
X3	1.90	98.5
X4a	2.44	72.7
X4b	2.58	68.1
AC	3.80	42.3
TM	4.36	35.8
X5	4.71	32.6

Same SDS-PAGE diagrams shown in Figure 1 were used for estimation of relative mobility of each component. Molecular weight of protein components in the heat induced gel was assumed by using a standard protein marker (Thermo Scientifics Co.).

Results

Solubility of heat-induced gel proteins in various solvents

Figure 1 shows the solubility of protein components from directly heated- and two-step heated gels of greenling and pollack, with and without the addition of albumen, in various solvents. The order of protein solubility was S1<S2<S3<S4<S5, regardless of the fish species or the presence or absence of albumen. Although preheating had little influence on the solubility of greenling heat-induced gel proteins, it resulted in decreased pollack protein solubility

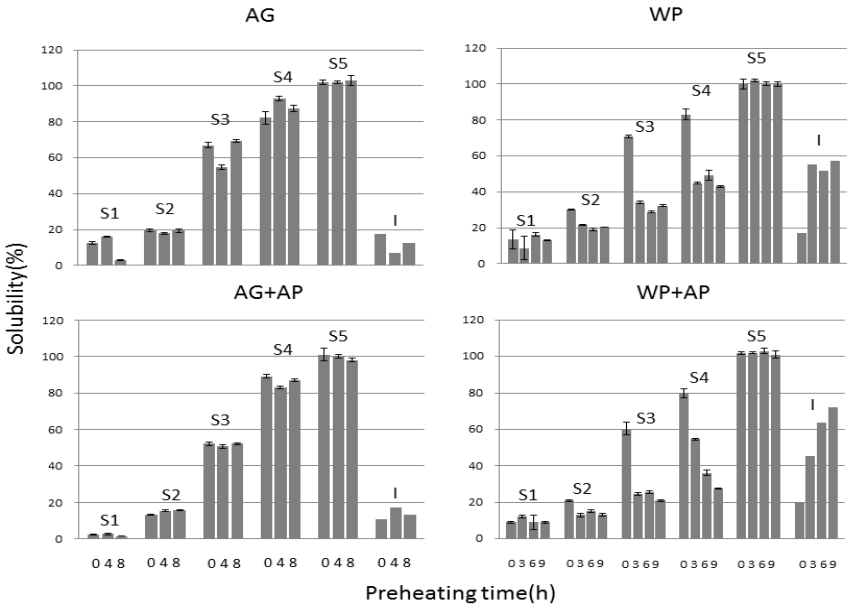

Figure 1. Protein solubility in various solvents of heated gels, formed from frozen surimis with and without addition of albumen powder, as a function of preheating time

The NaCl (3%)-ground meat with and without addition of 3%albumen powder from frozen surimi was heated at 90% for 30min with and without through the preheating at 25% for several hours. The heated gel was minced and solubilized in five solvents by continuous stirring at room temperature for 48 hrs. The soluble protein in the solvent was precipitated on addition of a final concentration of 7.5% TCA. The protein was dissolved in 1M NaOH and determined quantitatively by a Biuret test.

Protein solubility (%) = (soluble protein in solvent / total protein in salt-ground meat) ×100

The content (%) of insoluble (I) protein in the heated gel was calculated by subtracting S4 from 100.

All determination was performed in triplicate and all the data was expressed as mean ± standard deviation.

Solvent :

S1, 0.6 M NaCl

S2, 0.6 M NaCl + 1.5 M urea

S3, 0.6 M NaCl + 8.0 M urea

S4, 0.6 M NaCl + 8.0M urea + 2%mercaptoethanol

S5, 2.0% SDS + 8.0 M urea + 2% mercaptoethanol

I, Insoluble protein (100-S4)

Frozen surimi :

AG, arabesque greenling ; WP, walleye pollack

Additive :

AP, 3% albumen powder

in S2, S3, and S4 as well as resulted in increased insoluble protein component in solvents S1–S4. This strongly suggests that the protein structure of two-step heated gels clearly differs between the fish species used for the surimi, that is, the protein composition and the types of intermolecular forces contributing to heat-induced gel structural formation vary between fish species. Therefore, we proceeded to analyze the composition of fish-derived proteins dissolving in solvents S1–S5 and their changes caused by preheating, and compared between the heat-induced gel types.

Composition of heat-induced gel proteins dissolving in various solvents

Compositions of heat-induced gel proteins dissolving in various solvents were analyzed with SDS-PAGE using 7.5% polyacrylamide gel. The electrophoretograms and their image analysis results are shown in Figure 2 and 3, respectively. As shown in Figure 2, the main protein components of greenling dissolving in S2 were those corresponding to categories X4a and tropomyosin with X4b and X5 in smaller quantity, accompanied by minute quantities of Xn, MHC, and X1. Electrophoretograms also revealed that this did not change greatly with the

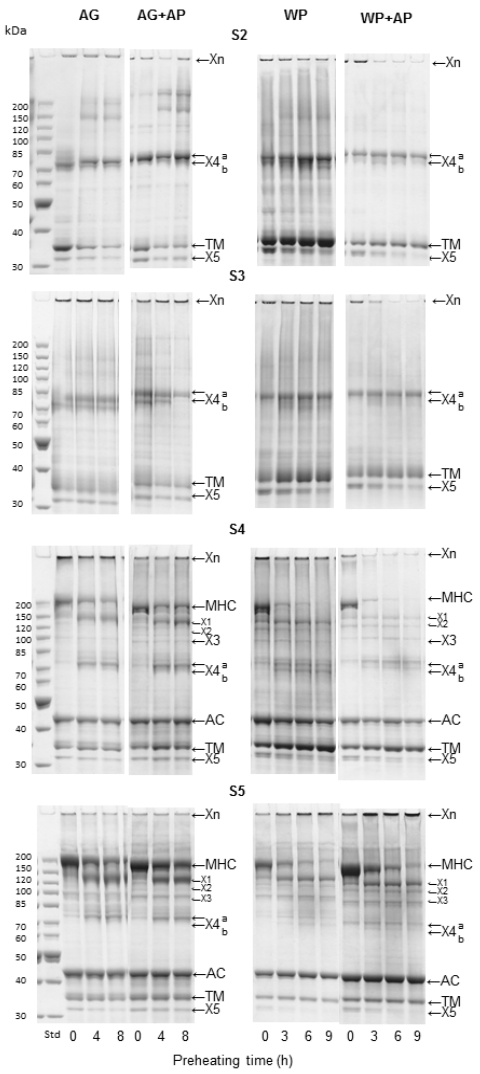

Figure 2. Change in SDS-PAGE diagrams of soluble protein of heated gels formed from frozen surimi with and without addition of albumen powder as a function of preheating time

The same heated gels as in Figure 1 were minced and solubilized in various solvents. Soluble proteins (10μg) were analyzed by SDS-PAGE using a 7.5% polyacrylamide slab gel. Protein bands were stained with Coomassie Brillant Blue-R250.
Frozen surimi:
AG, arabesque greenling
WP, walleye pollack
Additive:
3% albumen powder (AP)
Solvent:
S2, 0.6 M NaCl + 1.5 M urea
S3, 0.6 M NaCl + 8.0 M urea
S4, 0.6 M NaCl + 8.0 M urea + 2% mercaptoetanol
S5, 2% SDS + 8.0 M Urea + 2% mercaptoetanol
All solvents contained 20 mM Tris-HCl (pH 7.5)
Protein components:
Xn: Unidentified component, not migrating into gel
MHC: Myosin heavy chain
X1~5: Unidentified components (Component X4 was subdivided into X4a and X4b, as it was comprised in some cases of two components of close molecular weight.)
AC: Actin
TM: Tropomyosin

preheating time (4–8 h). Specific changes revealed by the image analysis (Figure 3a) were increased in X4a and trends of decrease in X4a and tropomyosin with preheating time, which suggest the occurrence of minor biochemical changes with preheating. As in greenling, the main components of pollack heat-induced gel dissolving in S2 were categories X4a, tropomyosin, and X5 (Figure 2). Component Xn was also observed, but no notable changes were observed with the preheating time (3–9 h). These observations were consistent with the image analysis results (Figure 3c). In the heat-induced gels with albumen, the main protein components dissolving in S2 were X4a, tropomyosin, and X4b, X5 for both greenling and pollack, as in the counterparts without albumen (Figure 2). However, image analysis revealed the notable presence of component Xn in pollack with albumen, which seemingly diminished over preheating time (Figure 3d). Other differences in pollack protein composition caused by the addition of albumen were slight increases in the amounts of dissolved X4a and tropomyosin. In greenling, the addition of albumen resulted in a decrease in X4b with preheating time (Figure 3b). Despite these many differences, the solubility of heat-induced gel proteins in solvent S2 remained between 15–20%, unaffected by the difference in fish species, the presence of albumen, or the preheating time (Figure 1). Therefore, the individual content of the dissolved protein components X4a, X4b, tropomyosin, and X5 would each amount to merely a few percent.

The composition of protein components dissolving in S3 closely resembles that of S2, appearing almost identical. That is, the main components were those corresponding to categories X4a and tropomyosin with a small amount of X4b and X5, accompanied by minute amounts of Xn, X1, and X3. Protein components dissolving in S2 are mainly those binds to the heat-induced gel structure by ionic bonds and hydrogen bonds, whereas protein components dissolving in S3 are those held together by ionic bonds, hydrogen bonds and hydrophobic interactions. Thus, it became clear that no additional components besides X4a, X4b, tropomyosin, and X5 could be solubilized by severing the hydrophobic interactions. However, the component categorized as Xn was seemingly augmented in heat-induced gels of both greenling and pollack. Moreover, component Xn of pollack in S3 showed a similar trend to its counterpart in S2, in which the addition of albumen resulted in its decrease with the preheating time. Solubility of heat-induced gel protein in S3 increased compared to that in S2, as shown in Figure 1. In directly heated gels, this increase in solubility amounted to

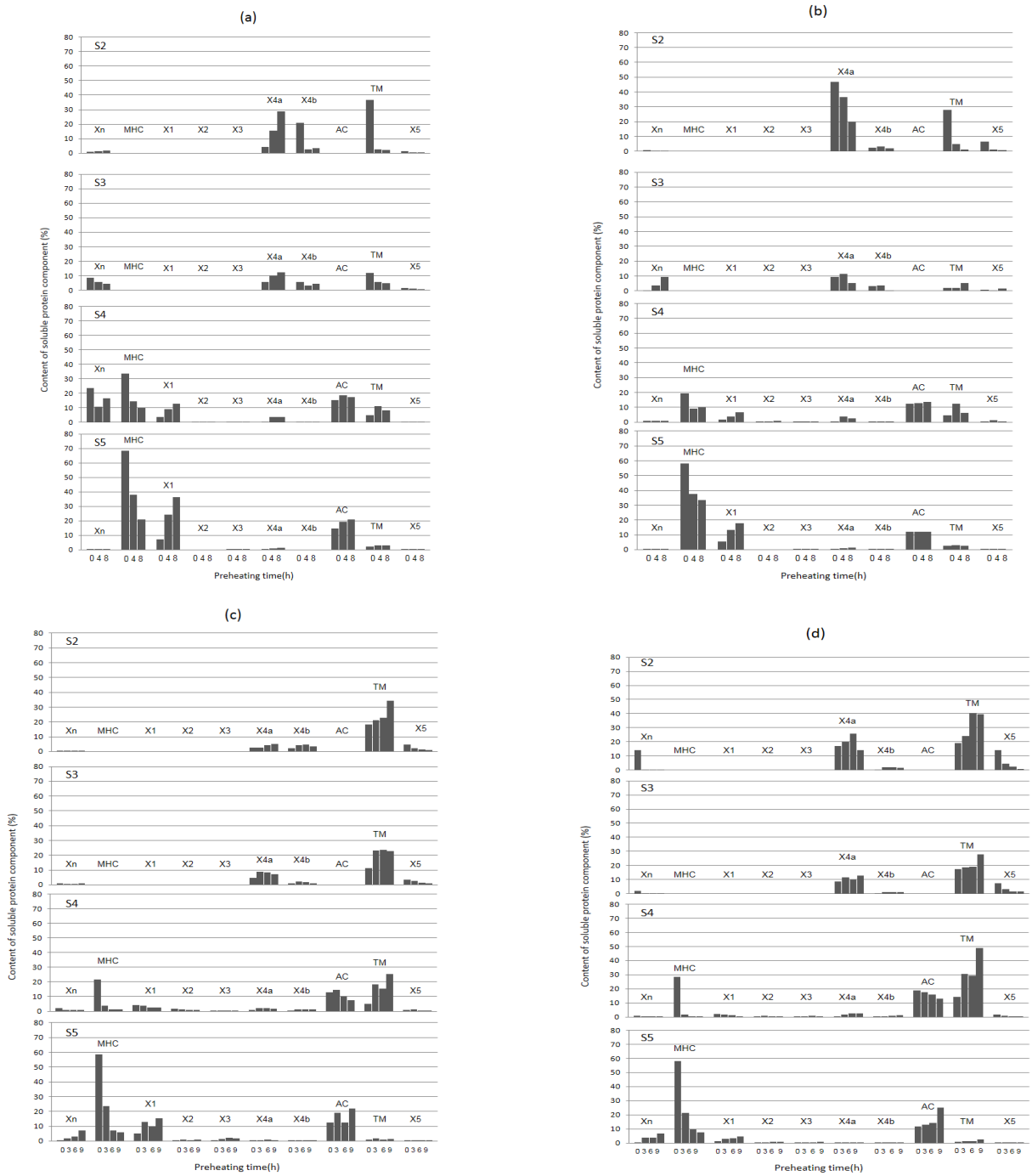

Figure 3. Change in content of soluble protein components in various solvents, of heated gels formed from frozen surimis with and without addition of albumen powder, as a function of preheating time

The same results of SDS-PAGE diagrams as in Figure 2 were scanned by using CanoScan LiDE 700F (Canon Co.) and digital images were imported into personal computer. The content of separated protein component was demonstrated as a relative intensity(%) of each band to that of all components.

Frozen surimi :

AG (a), AG + AP (b)

WP (c), WP + AP (d)

Additive :

AP, 3% albumen powder

Protein components :

Xn, X1~5, Unidentified components (Component X4 was subdivided into X4a and X4b, as it was comprised in some cases of two components of close molecular weight.)

MHC, Myosin heavy chain

AC, Actin

TM, Tropomyosin

The solvents and symbols used were the same as in Figure 2.

1.5–2.5 times, regardless of the fish species, which was slightly decreased with the addition of albumen. Preheating did not influence the solubility in S3 for greenling, whereas it was greatly reduced (halved) for pollack. However, the composition of eluted protein components and their ratios in S3 closely resembled those of S2 for both fish species as shown in Figure 2, with both mainly consisting of X4a, tropomyosin, X5, and Xn.

As shown in Figure 1, protein solubility in S4 reached high values of approximately 80% for directly heated gels, regardless of the fish species. This was slightly reduced in AG with the addition of albumen. Unlike in greenling, for which preheating did not have any significant influence, in pollack, solubility was greatly reduced by preheating. Figure 2 shows that S4-soluble proteins consisted of many components such as Xn, MHC, X1, X2, X3, X4a, X4b, actin, tropomyosin, and X5, of which Xn, X2, X3, and X5 were present in relatively small amounts. Moreover, components X4a, X4b, tropomyosin, and X5 were common to those in S2 and S3 as well. Thus, the components eluting selectively in S4 were MHC, X1, and actin for directly heated gels of both fish species. In two-step heated greenling gels, prolonged preheating reduced MHC; at the same time, the component corresponding to category X1 increased (or was generated). In contrast, X1 was present in only minute quantity in pollack and was not significantly augmented by preheating. Moreover, such differences in reaction to preheating were not significantly influenced by albumen. These series of changes in protein compositions were confirmed in Figure 3, but image analyses also showed the following characteristic changes: Xn was released in relatively high amount from greenling, which was reduced with the addition of albumen; and prolonged preheating resulted in a trend of increased tropomyosin in pollack.

Heat-induced gel proteins dissolved almost completely in S5 (Figure 1). This is because S5 contained SDS, which has a strong affinity to proteins and is effective in solubilizing them. Most kamaboko products dissolve well in solvents containing SDS, except for those with strongly crosslinked polymer structures that are artificially induced by microbial transglutaminase (Abe, 1998). Figure 2 revealed that the composition of protein components dissolving in S5 closely resembled that of S4, but for several points. The staining intensities of the main components in greenling such as MHC, X1, tropomyosin, and actin seemingly intensified compared to that of S4. The same was observed for pollack as well, but with an additional notable presence of what was assumed to be MHC polymers on the upper-most part of electrophoretogram. Moreover, preheating resulted in decreased Xn and MHC and increased X1 in greenling; in pollack, MHC was decreased to an even greater degree with only a small increase in X1 and a notable increase in Xn instead. Image analyses of S5 revealed that MHC and X1 contents largely increased relative to those in S4, actin remained similar, and the other components generally decreased compared to S4 for both greenling and pollack. In addition, the overall trend of the effect of albumen in S5 (such as the general decrease in X1) was similar to that in S4 for both greenling and pollack; however, a unique trend of increase in Xn was observed with preheating in S5 for pollack, which was seemingly enhanced with the addition of albumen. For greenling, Xn was hardly detectable in S5. The component categorized as Xn was also observed among the proteins eluted in S2, S3, and S4 for both fish species, but Xn did not increase with preheating in any of these solvents. Moreover, the addition of albumen mostly resulted in the decrease of Xn in those solvents, especially in pollack, but slight increase of Xn exceptionally in S3 in the case of greenling.

As SDS-PAGE with 7.5% polyacrylamide slab gel is unsuitable for analyzing protein components with large particle size, we repeated the analysis using 5.0% polyacrylamide slab gels. However, the component categorized Xn still did not migrate, and was stained in an accumulated state at the top of the polyacrylamide gels (data not shown). Thus, we were still unable to perform a precise quantification of the protein components. However, component Xn included in the protein components of heat-induced gels eluting in solvents S2, S3, and S4 was not influenced by preheating, regardless of the fish species. Only in the case of pollack, the addition of albumen had a general decreasing effect on the quantity of Xn. However, component Xn of greenling in solvent S5 was greatly reduced compared to the other solvents, appearing almost completely absent, whereas the same component of pollack in S5 showed a clear trend of increase with the preheating time. Therefore, there is a possibility that the component Xn eluting in S5 comprises two different types of proteins: one that is not influenced by preheating or albumen, and another does increase with preheating and is augmented by the addition of albumen.

Discussion

In this study, we analyzed the solubility and composition of heat-induced gel proteins in various types of solvents (S1–S5) using SDS-PAGE. From

these results, we determined the main protein components and the types of intermolecular forces by which those proteins are involved in heat-induced gel structures, and contemplated their roles in the formation of suwari heat-induced gel as well as the structure-reinforcing effect of albumen.

Proteins dissolving in S2 most likely correspond to those surimi-derived protein constituents that chiefly participate in the formation of heat-induced gel protein structure via ionic bonds (electrostatic interactions) and hydrogen bonds. Components Xn, X4a, X4b, tropomyosin, and X5 were detected from all heat-induced gels of both fish species; presumably, these components are involved in the heat-induced gel structure through relatively weak intermolecular forces. Furthermore, as these components were extracted uniformly from heat-induced gels of both fish species formed with or without suwari, they are probably unrelated to the specific physical properties of each heat-induced gel type. The main component, categorized as X4a, X4b comprises two subtypes of similar molecular weights that remain unidentified. Because these components are common to heat-induced gels of both fish species, it is highly likely that they belong to the myofibrillar proteins (regulatory proteins) (Yasui, 1993), or belong to the sarcoplasmic proteins that could not be removed from surimi by conventional washing process (Arnold *et al.*, 1968). In any case, the total amount of protein dissolving in S2 amount to, at most, 15–20% in heat-induced gels of both fish species, thus the content of the partial component X4 (X4a+X4b) must be of even smaller value (Kunimoto *et al*., 2014). Accumulation of a high molecular weight–component (Xn) was observed on the top-most edge of the polyacrylamide gel, but because this component was visible in low quantities and was not influenced by preheating, it is most likely derived from fish collagens located in the residual scales and skin in surimi (Mizuta, 2001).

Protein components eluting in S3 presumably correspond to those involved in the heat-induced gel protein structure formation via ionic bonds, hydrogen bonds, and hydrophobic interactions. Protein solubility was high in S3, reaching approximately 60-70% for heat-induced gels of both fish species, although this was slightly decreased by albumen, but the composition of eluted components were very similar to that of S2 with the main components consisting of Xn, X4a, tropomyosin, and X5. It is therefore plausible that that these components are kept in place partly by relatively weak intermolecular forces that are breakable by solvent S2, and partly by somewhat stronger forces that are breakable by S3 but S2, regardless of the fish species and suwari phenomenon. In the latter case, hydrophobic interactions are probably this additional intermolecular force.

The protein components dissolving in S4 likely correspond to these constituents released from the heat-induced gel protein structure when disulfide bonds is severed in addition to ionic- and hydrogen bonds, as well as hydrophobic interactions (those region disrupted by 8.0 M urea). This suggests that disulfide bonds is important for those protein components released in S4 but not for those in S2 or S3 to participate in the heat-induced gel protein structural formation. In directly heated gels of both greenling and pollack, components MHC and actin corresponded to this description. In two-step heated gels, however, fish species-specific differences were seemingly observed, with components X1 and MHC being the S4-specific eluents for greenling and components X1 and actin being that for pollack. The component categorized as X1 has not yet been identified; however, as it seems to be generated with the decrease in MHC, it may be a degradation product of MHC via endogenous enzyme activity (Ichishima, 1983; Konno *et al.*, 2000). The molecular weight of component X1 resembles that of heavy meromyosin-like product, but we have not been able to confirm this identity yet because of its lack of tendency to increase with preheating. Component X1 was present only in low quantity for pollack, and the detected amount did not match the decrease in MHC. This is probably because MHC was catabolized into substances that are less soluble in S4, which were in turn detected in S5.

As S4 comprised NaCl, urea (8.0 M), and Me, it cleaved not just the disulfide bonds, but also other intermolecular forces between proteins. We have therefore separately measured and compared the protein solubility in solvents containing NaCl, urea, or Me alone, which revealed that solubility of proteins from directly heated gels was around 10% in a solvent containing NaCl alone, approximately 10–15% for Me alone, and about 55% for urea, regardless of the fish species. The solubility increased moderately in Me and decreased only slightly in urea for pollack with the addition of albumen. It is therefore probable that the part of MHC, X2, and actin that dissolved in S4 contribute to the heat-induced gel protein structure formation via the concerted contribution of disulfide bonds and other forces, such as hydrophobic interactions. In the two-step heated gels, the solubility remained similar to the directly heated gel for greenling. For pollack, the solubility in NaCl and Me was similar to that of directly heated gel, but decreased to 50% or lower in

urea. This was then further halved with the addition of albumen, suggesting that the insolubility of protein structure was enhanced through the involvement of intermolecular forces that are stronger than disulfide bonds.

Presumably, the component categorized as Xn among the proteins dissolving in S5 is different from its counterparts in S2–S4; numerous past findings indicate that it corresponds to a polymer of MHC (Numakura *et al*., 1987). We were unable to quantify the changes in this component presumed to be MHC polymer (MHCn) with the electrophoresis conditions employed in this study. However, it has been previously reported that MHCn is produced in two-step heated gels of pollack by an exceptionally strong bonds force (Numakura *et al*., 1987; Kimura, 1991) whenever there is a rapid decrease of MHC solubility in S4 due to preheating (Kunimoto *et al*., 2014). It has also been reported that such changes in protein components are seen in gel formation with suwari, and are strongly implicated in the significant increase of physical property values over time (Kunimoto *et al*., 2014).

The hypothesis that proteins originating from albumen contribute to the formation of heat-induced gels via disulfide bonds has been reported previously (Kunimoto *et al.*, 2014). Measurements of protein solubility from heat-induced gels formed with albumen alone (volume of water increased to 7 times (v/w) and heated for 30 min at 90°C) revealed that the solubility in S1, S2, and S3 were all around 10%, in the order S1<S2<S3. As the solubility was 52% in S4 and 95% in S5, it is presumed that the heat-induced gel of albumen itself is constructed with strong disulfide bonds. SDS-PAGE analysis of proteins dissolving in S5, performed in a manner similar to Figures 1 and 2 in this study, has revealed the presence of two types of components with molecular weight of about 70–85 kDa and 40 kDa (not shown in the figure). These molecular weights closely resemble those of conalbumin and ovoalbumin, respectively (Cheftel *et al.*, 1988). The presence of these two components among those proteins dissolving in solvents S2 and S3 was not clear; instead, the presence of a component corresponding slightly to Xn was observed. Because Xn was not found in S4 and S5, which contain Me, it could be an albumen-derived multimer. The presence of the two protein components derived from albumen was not confirmed among the proteins that dissolved in S4 and S5 from heat-induced gels prepared with additive albumen, however, this is thought to be due to the low quantity of additive (3%).

To sum up these results, the primary protein components of surimi participating in the formation of heat-induced gel protein structure seemed to be MHC and actin. In greenling, MHC and its derivative X1 (mainly MHC in directly heated gels, and MHC and component X1 in two-step heated gels to be precise) contributed to the structure via strong disulfide bonds and hydrophobic interaction together with actin. In pollack, however, it was suggested that MHC and MHCn (mainly MHC in directly heated gel and MHCn in two-step heated gels) contributed to the structure via an extremely strong intermolecular force (combined effect of strong hydrophobic interactions, disulfide bonds, and possibly other bonds such as isopeptide bonds (Kimura, 1991), together with actin.

Part of the components such as Xn, X3, X4a, X4b and X5 are suggested to bind to the major protein components of surimi (e.g. MHC and actin) via ionic and hydrogen bonds, etc. Contribution of these components to the protein structure of heat-induced gels is considered to be negligible, as they were present in both greenling and pollack in relatively minute quantities and were not influenced by preheating.

Conclusion

In the case of greenling, the main proteins contributing to the heated gel structure were AC and MHC for directly heated gels, and actin, MHC and component X1 (140.2 kDa) for two-step heated gels. In either gel type, the formation of gel structure was suggested to involve strong disulfide bonds and hydrophobic interactions. However, in the case of pollack, the gel structure formation was suggested to involve AC and MHC in directly heated gels and AC and MHC polymer in two-step heated gels, through the concerted action of strong hydrophobic interactions, disulfide bonds, and possibly also isopeptide bonds. These results indicated that main protein components contributing to the formation of heat-induced gel were largely depend on fish-species used for making frozen surimi. Especially, there was considerable difference between the two-step heated gels of AG and WP formed through suwari-gel setting process.

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