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Research Article

Effects of Freeze-Thaw Cycles on Gel-Forming Ability and Protein Denaturation in Alaska Pollock Frozen Surimi

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Frozen surimi may be damaged by freeze-thaw cycles (refreezing) under various conditions. However, few studies have examined the deterioration of the quality of refrozen surimi. The objective of this study was to determine the deterioration mechanism of refrozen surimi. We used Alaska pollock frozen surimi, which has been studied extensively for gel formation. Refreezing decreased the breaking strength and breaking strain of the heated gel. The length of the line in the diagram between breaking strength and gel stiffness (L value, which indicates the level of change in the breaking strength and gel stiffness with setting time) was also decreased. In contrast, the effect of refreezing on the texture of the gel without the setting process was small. The polymerization rate of myosin heavy chain in refrozen surimi during the setting process was slower than that in nonrefrozen surimi. Additionally, the Ca-ATPase activity of surimi was reduced by approximately 30% with each refreezing. These results demonstrate that the reduction in gel-forming ability by refreezing was caused by the decreased polymerization rate of myosin heavy chain because of myosin head denaturation. We also found that quality deterioration including myofibrillar protein denaturation of frozen surimi occurred mostly during first refreezing rather than during second refreezing. Overall, refreezing and/or repeated refreezing of surimi simply decreased the suwari gel-forming ability without changing the characteristics of surimi. The primary cause of the decrease in gel-forming ability induced by refreezing is considered to be the suppression of myosin heavy-chain polymerization during setting.

1. Introduction

It is well known that refreezing reduces the quality of food products [1, 2]. Refreezing causes damage such as cell destruction, protein denaturation, and surface drying in foods. While many studies have examined the effects of refreezing on food products, few reports have evaluated food materials.

Surimi paste products are important components of seafood products. The main material in surimi paste products is frozen surimi. The most important property of frozen surimi is its gel-forming ability, which depends on the structure of myofibrillar proteins in surimi. Kato et al. [3] detected a correlation between the Ca-ATPase activity of frozen surimi and gel-forming ability. Additionally, denatured myofibrillar protein in surimi cannot form gel [4]. Myofibrillar protein in fish muscle is susceptible to denaturation by heating and/or freezing because of its sensitivity to changing temperatures [5]. In most previous studies, the gel-forming ability was evaluated by conducting a texture test. In Japan, the punch test has been used to evaluate the gel-forming ability of frozen surimi. Abe et al. [6] found a linear relationship between breaking strength and gel stiffness. Additionally, they revealed that the line position, linear slope, and plot dispersion depend on the property of frozen surimi. Figure 1 shows a model of the relationship between gel stiffness and breaking strength of two-step heated gels. The relationship diagram of breaking strength versus gel stiffness revealed the following. (1) When the straight line was shifted to the right side or when its incline was small, the surimi formed a brittle and hard gel. (2) When the plots were scattered, the surimi formed a nonuniformed gel. For example, the plots of suwari gel with microbial transglutaminase tended to be scattered. (3) When the plots formed clumps, the surimi did

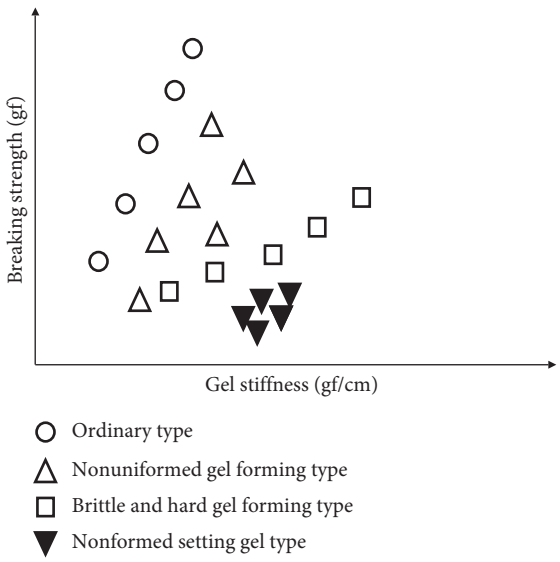

Figure 1: Model of GS versus BS relationship diagram. ○, △, □, and ▼ indicate various types of surimi.

not form a suwari gel. These relationships between breaking strength and gel stiffness have been used to analyze the gel-forming ability of surimi [6–9].

Frozen surimi is manufactured in many countries. However, frozen surimi factories are at risk of unexpected temperature rise of frozen surimi. Particularly, the warm climate is one factor causing a rise in the temperature of frozen surimi, which can damage the product when thawing and freezing (refreezing) go unnoticed. Furthermore, refreezing after a temperature increase may occur during transportation. Thus, the refreezing risk of frozen surimi is an important problem in the surimi paste product industry. Several effects of refreezing on the gel-forming ability of frozen surimi have been reported [10–12]. Another study showed that refreezing of frozen surimi reduces its gel-forming ability [13]. Furthermore, ionic and hydrogen bonds are increased in heat-induced gel prepared from refrozen surimi, whereas hydrophobic interactions, S-S bonds, and more intensive bonding interactions are decreased [13]. However, the refreezing problem of frozen surimi in the surimi seafood industry has not yet been solved. To address this problem, previous studies examined the effects of refreezing times that cannot occur at production sites. Moreover, these previous reports did not fully consider the reduction of the gel-forming ability by refreezing, i.e., these reports did not study the change in the suwari gel-forming ability with setting time. Therefore, the purpose of this study was to determine the effect of refreezing on the relationship between protein denaturation and gel-forming ability of frozen surimi after different setting times.

2. Materials and Methods

2.1. Materials. Alaska pollock (*Theragra chalcogramma*) frozen surimi (A grade) was obtained from Maruha Nichiro Corporation (Tokyo, Japan) and then cut into approximately 600 g blocks. The cut frozen surimi was stored at −30°C in a freezer until further use. The refrozen surimi was prepared by freezing at −30°C for at least 24 h after thawing at 4°C overnight (1st F-T cycle surimi). The refreezing process was repeated twice (2nd F-T cycle surimi) within 1 week. Potassium chloride, calcium chloride, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), maleic acid, trichloroacetic acid, 2-mercaptoethanol, dodecyl sodium sulfate (SDS), urea, and bovine serum albumin were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of Heat-Induced Gel. Frozen surimi blocks were thawed by refrigeration (4°C) overnight and then cut into small cubes. Surimi cubes were ground with water (60 mL/600 g of surimi) until the surimi cube temperature was 1–2°C. Next, 3% NaCl was added and grinding was continued until the surimi paste temperature was 7–10°C. The surimi paste was stuffed into a polyvinylidene chloride tube (diameter, 24 mm). Next, the tubes containing surimi paste were preheated for 0–24 h at 20°C in a water bath (suwari gel). At set time points, some tubes were removed from the water bath and heated for 30 min at 90°C. After heating, the gels were chilled immediately in water for over 10 min. These gels were defined as two-step heated gels. The gel heated for 30 min at 90°C without preheating was defined as the directly heated gel.

2.3. Gel Texture Measurement. All gels were cut into pieces of 3.0 cm in length and 2.5 cm in diameter. Breaking strength and breaking strain of the gel samples were measured with a creep meter (RHEONER II, Yamaden, Tokyo, Japan) using a spherical plunger that was 5 mm in diameter with a depression speed of 1 mm/s. Gel stiffness was calculated using the following equation (1):

$$\text{gel stiffness (g/cm)} = \frac{\text{breaking strength (g)}}{\text{breaking strain (cm)}}. \quad (1)$$

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The sample used to analyze the protein patterns of heat-induced gel by SDS-PAGE was prepared as described by Numakura et al. [14] with slight modifications. Gels were added to SDS-urea solution containing 2% SDS, 2% 2-mercaptoethanol, 8 M urea, and 20 mM Tris-HCl (pH 8.8) and heated to 100°C for 2 min. The sample was shaken to dissolve the gel in SDS-urea solution on a seesaw shaker overnight. The solution was applied to SDS-PAGE by the Laemmli method [15] using 7.5% separating polyacrylamide gels (e-PAGEL®, Atto, Tokyo, Japan). The gel was stained using Coomassie Brilliant Blue.

2.5. Determination of Residual Rate of Myosin Heavy Chain (MHC) and the Concentration of 150 kDa Band. The image data of SDS-PAGE pattern were obtained using a scanner. The staining intensity of MHC band and the 150-kDa band

was determined by Image J. The rate of staining intensity of these two bands was calculated by dividing their staining intensity by the staining intensity of all bands. The MHC residual rate was calculated assuming that the concentration of the band with a setting time of 0 hours was 100%.

2.6. Preparation of Myofibrillar Protein Suspension from Frozen Surimi. The myofibrillar protein suspension of frozen surimi was prepared as described by Kato et al. [16] with slight modifications. Thawed surimi was added to 0.1 M KCl and 20 mM Tris-HCl (pH 7.0) and then homogenized. The homogenate was centrifuged at $7{,}500 \times g$ for 20 min. After removing the supernatant, 0.1 M KCl and 20 mM Tris-HCl (pH 7.0) were added. This process was repeated until the supernatant was clear. The precipitate was suspended in 0.1 M KCl and 20 mM Tris-HCl (pH 7.0) and then filtered through a gauze. The filtrate was used as the myofibrillar protein suspension. There was almost no change in the recovered amount of myofibrillar protein from each frozen surimi. The prepared myofibrillar protein suspension was stored on ice until measurement of Ca-ATPase activity.

2.7. Measurement of Ca-ATPase Activity. The myofibrillar protein suspension (2.5–3.5 mg/mL) was added to 0.1 M KCl, 5 mM $CaCl_2$, 1 mM ATP, and 25 mM Tris-maleate (pH 7.0). The reaction was conducted at 25°C. The reaction was stopped by adding 15% perchloric acid at each reaction time. The supernatant was added to sulfate-molybdate acid solution and Elon reagent. The inorganic phosphate concentration in the supernatant was measured by colorimetric determination at a wavelength of 640 nm. Ca-ATPase activity was calculated using the following equation (2):

$$\text{Ca-ATPase activity} = \left\{\ln\left(1 - P_{i0}\right) - \ln\left(1 - P_{it}\right)\right\} \times \frac{1000}{t} \times \frac{1}{C_{\text{m}}} \left[\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg of protein}^{-1}\right], \quad (2)$$

where t is the time of reaction; P_{i0} is the inorganic phosphate acid concentration at a reaction time 0 min (blank); P_{it} is the inorganic phosphate acid concentration after the reaction; and C_{m} is the concentration of myofibrillar protein in the reaction reagent.

The protein concentration of the myofibrillar protein suspension used in the reaction was determined by the biuret method using bovine serum albumin as a standard.

2.8. Statistical Analysis. Statistical analysis was performed using the bell curve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). The results were compared using the least significant difference method at $p < 0.05$.

3. Results and Discussion

3.1. Gel Texture Measurement. Changes in the breaking strength and strain of suwari gel prepared from frozen surimi are shown in Figure 2. Compared to that of the control sample, the breaking strength of refrozen surimi was low. In contrast, the breaking strength of the 1st and 2nd F-T cycle samples showed nearly the same value. The breaking strain of the control sample was higher than that of the 1st and 2nd F-T cycle samples until a setting time of 3 h. However, all samples showed nearly the same value after 6 h of setting time. Figure 3 shows the relationship between breaking strength and gel stiffness of the suwari gel prepared from each frozen surimi sample. When the plots formed clumps in the relationship diagram of breaking strength versus gel stiffness, the surimi did not form a suwari gel [8]. Therefore, the length of the line in the relationship diagram of breaking strength versus gel stiffness indicates the level of change in the breaking strength and gel stiffness with setting time. Thus, we considered that the length would be an indicator of suwari gel-forming ability. In this study, the length of the line was defined as the L value. The L value in Figure 3 is the simplified calculated length of an approximate straight line. The length was calculated by substituting the minimum point and maximum point, which were defined as the shortest and longest lengths from the origin point, respectively, into the equation for the approximate line. The relationship between breaking strength and gel stiffness was linear in the suwari gel prepared from all samples. Previous studies of surimi gel confirmed the linear relationship between breaking strength and gel stiffness [6]. We confirmed the linear relationship with the suwari gel. As shown in Figure 1, the plots of the surimi with poor suwari gel-forming ability formed clumps in the breaking strength versus gel stiffness relationship diagram. Therefore, the L value of the surimi that lost its suwari gel-forming ability was low. As shown in Figure 3, the L value decreased each time the refreezing was repeated. However, the slope and location of the line were largely unchanged by refreezing. This indicates that refreezing and/or repeated refreezing of surimi simply decreased the suwari gel-forming ability without changing the characteristics of surimi.

Changes in the breaking strength and strain of the direct and/or two-step heated gel prepared from frozen surimi are shown in Figure 4. The difference in breaking strength and breaking strain of the direct heated gel (i.e., setting time of 0 h) prepared for all samples was small. In contrast, the breaking strength and breaking strain value of the two-step heated gel prepared from the control sample was higher than that of those prepared from the refrozen sample. As observed for the suwari gel, the breaking strength and breaking strain of the two-step heated gel prepared from the 1st F-T cycle sample did not differ from those of the two-step heated gel prepared from the 2nd F-T cycle sample. As shown in Figure 4, the effect of refreezing on frozen surimi was confirmed to be low for surimi paste products produced without a setting process.

Figure 5 shows the relationship between breaking strength and gel stiffness of the direct and/or two-step heated

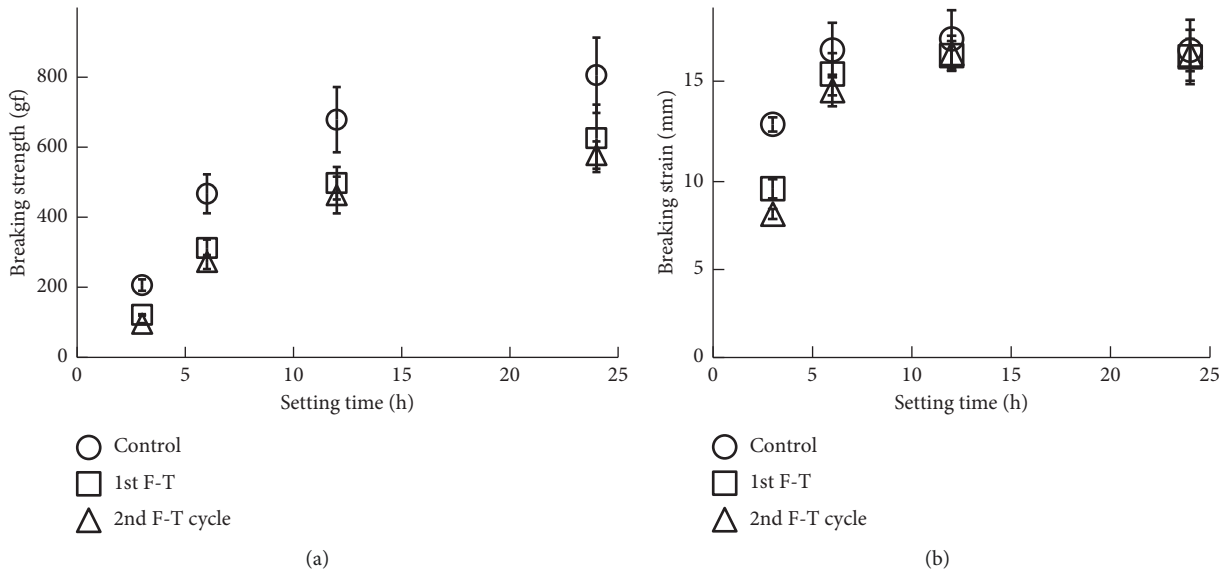

Figure 2: Changes in breaking strength and breaking strain of suwari gel prepared from each frozen surimi during setting time at 20°C. Error bar indicates standard deviation.

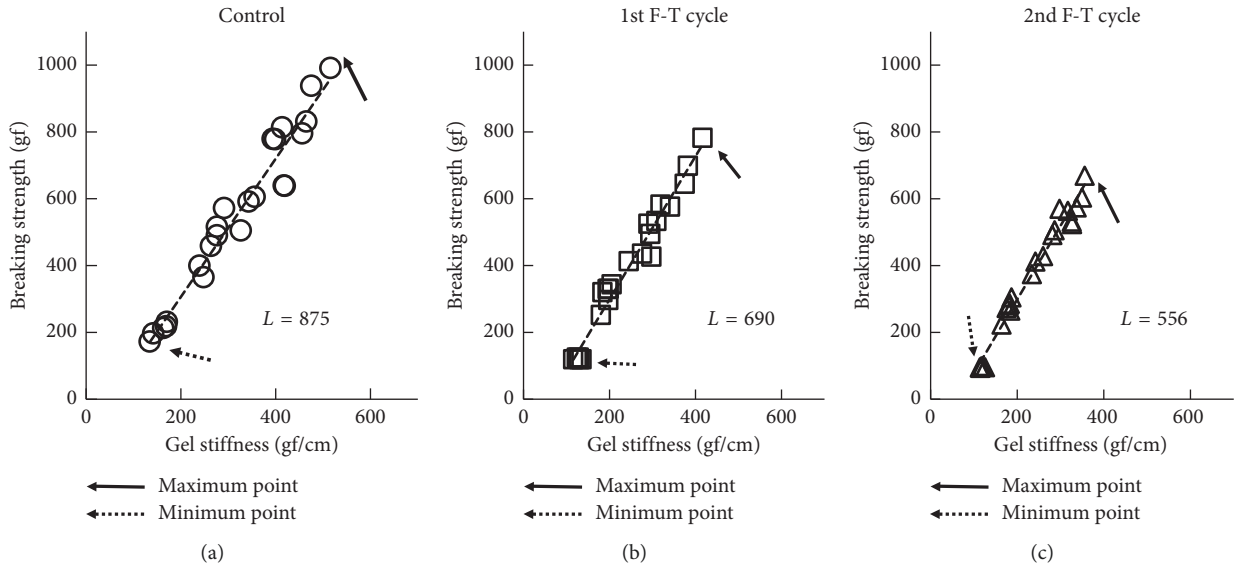

Figure 3: Relationship between breaking strength and gel stiffness of suwari gel prepared from each frozen surimi sample. *L* value indicates the approximate length of the straight line from the minimum point to the maximum point. Minimum point and maximum point were defined as the shortest distance and longest distance from the origin, respectively.

gel prepared from each frozen surimi sample. The definition of the *L* value is the same as that in Figure 3. The relationship between breaking strength and gel stiffness was linear in the heated gel prepared from all samples. The *L* values of the heated gel prepared from each surimi sample were higher than those of the suwari gel. The value of breaking strength and gel stiffness increased because of gelation induced by heating. The *L* value of the heated gel decreased each time refreezing was repeated. The reduction in the *L* value of the heated gel by refreezing was considered to be caused by the loss of suwari gel-forming ability.

Previous studies on the indicators of the suwari reaction rate focused only on breaking strength and did not consider the concept of breaking strain [17, 18]. In this study, the *L* value, which includes the breaking strain (i.e., gel stiffness is calculated by dividing breaking strength by breaking strain) in Figures 3 and 5, well reflects the results in Figures 2 and 4 as the first refreezing reduced the gel-forming ability more than the second refreezing. Therefore, the *L* value may serve as a good indicator of the suwari gel-forming ability.

3.2. SDS-PAGE Patterns and Changes in Staining Intensity of the MHC Band and 150 kDa Band. The SDS-PAGE patterns of suwari gel prepared from each surimi sample are shown in Figure 6. Furthermore, the reduction rate of MHC band with

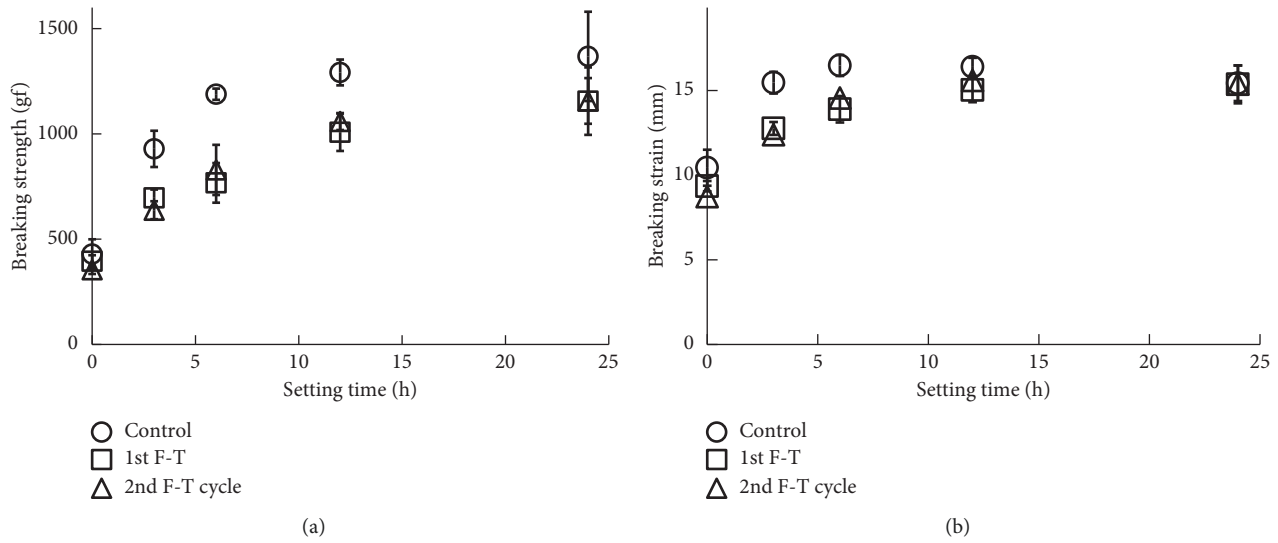

Figure 4: Changes in breaking strength and breaking strain of direct and/or two-step heated gel prepared from each frozen surimi during the setting time at 20°C. Error bar indicates standard deviation.

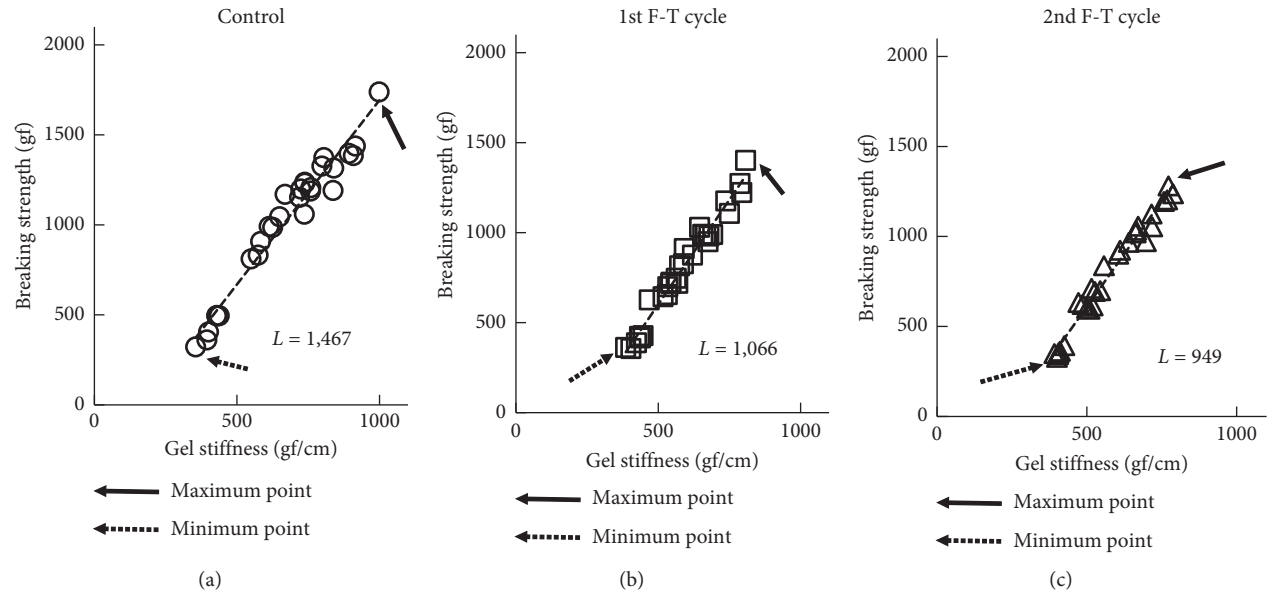

Figure 5: Relationship between breaking strength and gel stiffness of direct and/or two-step heated gel prepared from each frozen surimi sample. The L value indicates the approximate length of the straight line from the minimum point to the maximum point. Minimum point and maximum point are defined in Figure 3.

setting time is show in Figure 7. During setting, MHC formed a large polymer that could not be loaded onto the electrophoresis gel. Therefore, the MHC band of the suwari gel prepared from control surimi decreased for 6 h. In contrast, the MHC band of the suwari gel prepared from the 1st and 2nd T-F cycle surimi samples decreased more slowly than that of the control surimi. The decreasing rate of the MHC band in the suwari gel prepared from the 1st T-F cycle surimi was the same as that of the suwari gel prepared from the 2nd T-F cycle surimi. The SDS-PAGE patterns of the direct and/or two-step gel prepared from each surimi sample are shown in Figure 8, and the reduction rate of the MHC band with setting time is shown in Figure 9. The MHC band of heated gel prepared from control surimi rapidly decreased over the setting time. As observed for the suwari gel by SDS-PAGE, the MHC band of the heated gel prepared from the 1st and 2nd T-F cycle surimi samples decreased more slowly than that of control surimi. The delay of MHC polymerization by refreezing contributes to the decreases in the breaking strength and breaking strain and the reduction in the L value. The changes in the staining intensity of the 150-kDa band of suwari gel and two-step heated gel are shown in Table 1. The 150-kDa band appeared by setting. The staining intensity of the 150-kDa band tended to increase when

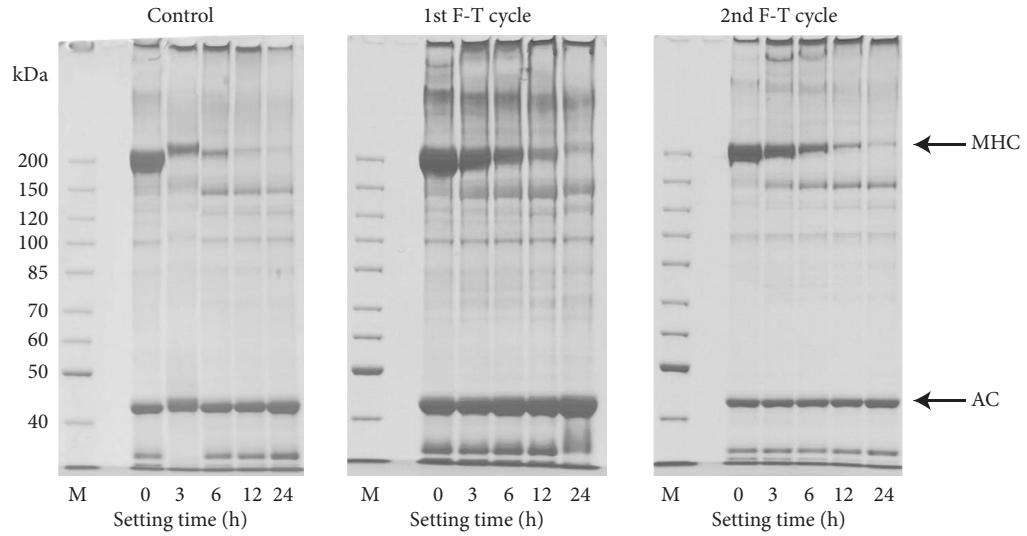

Figure 6: SDS-PAGE pattern of suwari gel prepared from each frozen surimi. M, MHC, and AC indicate the protein marker, myosin heavy chain, and actin, respectively.

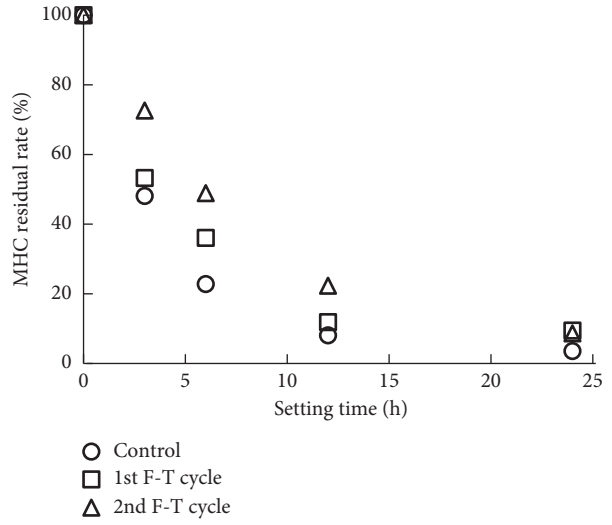

Figure 7: Changes in MHC residual rate of suwari gel prepared from each frozen surimi with setting time.

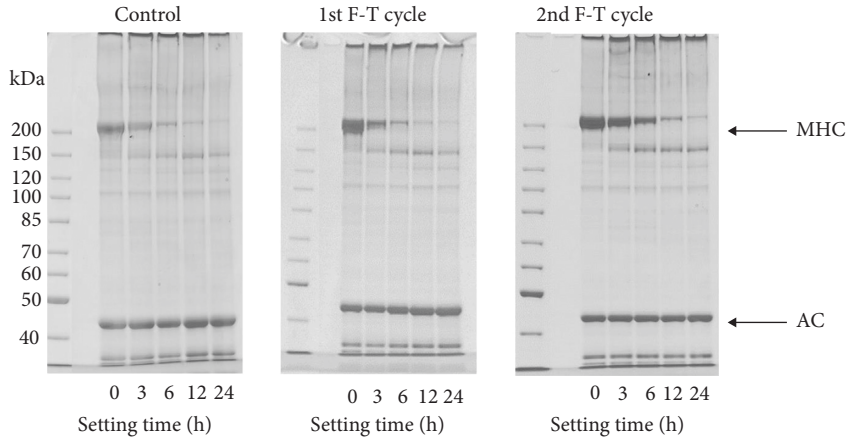

Figure 8: SDS-PAGE pattern of direct and/or two-step heated gel prepared from each frozen surimi. M, MHC, and AC indicate the protein marker, myosin heavy chain, and actin, respectively.

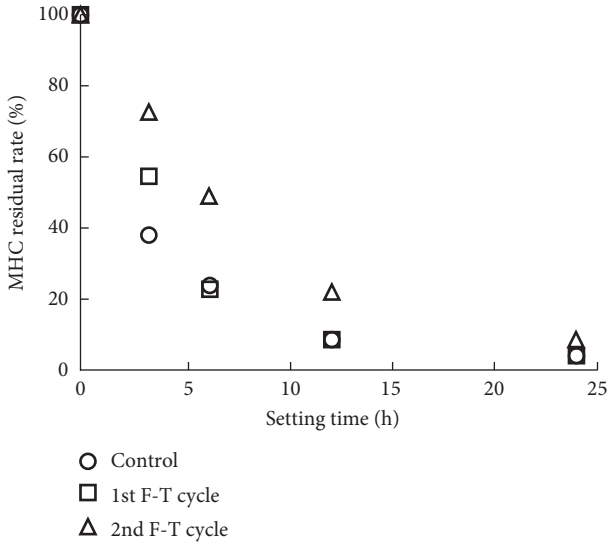

Figure 9: Changes in MHC residual rate of two-step gel prepared from each frozen surimi with setting time.

Table 1: The rate of 150 kDa band against all bands in SDS-PAGE of suwari gel and two-step heated gel.

Setting time (h)	Suwari gel			Two-step heated gel		
	Control	1st F-T cycle	2nd F-T cycle	Control	1st F-T cycle	2nd F-T cycle
0	0.6	0.7	2.8	3.7	2.4	3.2
3	6.1	5.9	8.1	5.6	6.5	6.9
6	10.9	7.1	11.4	7.4	8.1	10.7
12	9.2	9.2	10.4	9.2	10.7	13.2
24	8.2	11.7	12.3	9.1	9.3	13.0

refreezing was repeated. Konno and Imamura reported that the 150-kDa band appeared to be degraded by protease during setting [19]. Therefore, the MHC of refrozen surimi may be easily degraded by protease. Based on these results, the primary cause of the decrease in gel-forming ability induced by refreezing is considered to be the suppression of MHC polymerization during setting.

3.3. Changes in Ca-ATPase Activity of Surimi by Refreezing. Figure 10 shows the Ca-ATPase activity of each surimi sample, which decreased by approximately 30% with each refreezing. This demonstrates that the myosin heads were denatured by refreezing. In a previous study, the Ca-ATPase activity of surimi decreased linearly with each refreezing [20]. However, Ca-ATPase activity quickly decreased during the 1st refreezing and slowly decreased during the 2nd refreezing. This suggests that myofibrillar protein was strongly denatured by the 1st refreezing, greatly decreasing the quality of surimi based on the results of gel texture measurement and SDS-PAGE patterns. Therefore, denaturation of myosin heads by the 1st refreezing greatly reduces the suwari gel-forming ability. However, Ca-ATPase indicates only the denaturation of the myosin head. Thus, the myosin rod needs to be investigated to reveal the effect of refreezing on the gel-forming ability of frozen surimi.

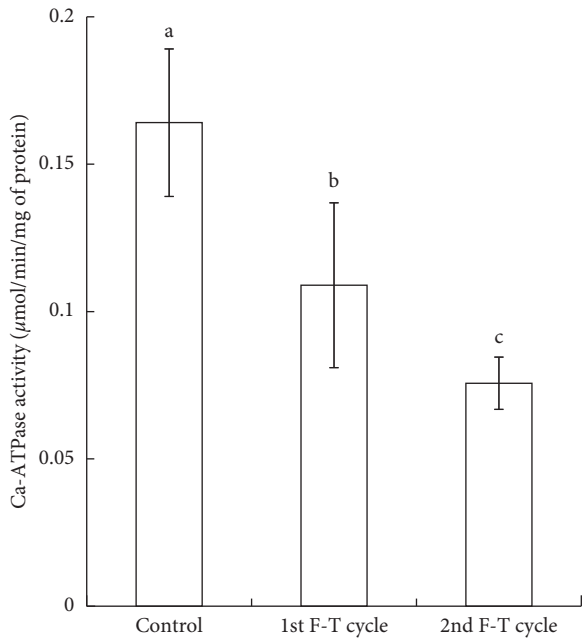

Figure 10: Ca-ATPase activity of each frozen surimi. Error bar indicates standard deviation. Different letters indicate a significant difference ($p < 0.05$).

4. Conclusions

In this study, the gel-forming ability of refrozen surimi was confirmed to be weakened by refreezing. In particular, the suwari gel-forming ability was greatly reduced by denaturation of the myosin head upon refreezing. In addition, MHC polymerization during setting was suppressed by refreezing. These findings revealed that decreasing the suwari gel-forming ability reduced the heated gel texture. Therefore, the influence of refreezing on the texture of surimi paste products without the setting process was small. The quality deterioration including myofibrillar protein denaturation of frozen surimi was greater during the 1st refreezing than during the 2nd refreezing.

Data Availability

The data used to support the findings of this study are included within the supplementary information files.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by JSPS KAKENHI (grant no. JP 15K16194).

Supplementary Materials

S-Figure 1(a): breaking strength raw data (setting gel); S-Figure 1(b): breaking strain raw data (setting gel). S-Figure 2(a): calculation process of gel stiffness (setting gel); S-Figure 2(b): correlation diagram between breaking strength and gel stiffness (setting gel). S-Figure 3(a): breaking strength raw data (double-heated gel); S-Figure 3(b): breaking strain raw data (double-heated gel). S-Figure 4(a): calculation process of gel stiffness (double-heated gel); S-Figure 4(b): correlation diagram between breaking strength and gel stiffness (double-heated gel). S-Figure 5 & Table 1: changes in MHC residual rate and 150 kDa band of suwari gel prepared from each frozen surimi with setting time. S-Figure 6 & Table 1: changes in MHC residual rate and 150 kDa band of direct and/or two-step heated gel prepared from each frozen surimi with setting time. S-Figure 7: Ca-ATPase activity raw data. (*Supplementary Materials*)

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