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Quality improvements to mackerel (Scomber japonicus) muscle tissue frozen using a rapid freezer with the weak oscillating magnetic fields

Kana Okuda [a,b], Aiko Kawauchi [a,b], Kentaro Yomogida [a,*]

[a] Department of Molecular Cell Biology, Institute for Bioscience, Mukogawa Women's University, Ikebiraki-cho 6-46, 663-8558, Nishinomiya, Japan
[b] Abi Inc., Ohtakanomori-higashi 1-12-1, 270-0138, Nagareyama, Japan

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ABSTRACT

Although freezing is the most popular long-term food preservation method, the formation of ice crystals during the freezing process often degrades the quality of the product. Recently, several reports have argued that oscillating magnetic fields (OMFs) may affect ice crystallization. In this paper, we investigated the effects of OMFs on fresh mackerel using the Cell Alive System® (CAS®) developed as an additional OMF generator for a rapid freezer. Mackerel fillets were frozen with home freezing (HF), air blast freezing without (ABF) or with CAS (ABF-CAS) (ABI Co. Ltd., Chiba, Japan), and stored them for 2 weeks in the frozen storage between −30 °C and −35 °C. We analyzed the tissue damages of thawed samples histologically. The OMFs has been shown to significantly inhibit tissue damage in mackerel tissue after freezing and thawing (especially, thawing in ice water). And it seems that OMFs suppressed the ice hole counts ($p < 0.05$), the mean size ($p = 0.061$), and the increase of interstitial area% ($p < 0.05$) after freezing/thawing. We also found that it is necessary to avoid re-crystallization during thawing to maintain the quality of the frozen product. The use of OMFs with rapid thawing has the potential to improve cryopreservation in the food industry as well as in the bioscience industry.

1. Introduction

The population explosion that has occurred in recent years is causing various global food security issues. To supply sufficient food to people all over the world, food production must increase while food loss and waste must be reduced. An improvement of food preservation technologies is also key to reducing food loss and waste. Freezing is the most popular and universal method of food preservation for the long term and is used both in the food industry as well as in ordinary household kitchens. Although freezing may well maintain the quality and taste of food products for a long period of time, the accumulation of microstructural damage caused by ice crystal formation during the freezing process often leads to degradation of food products [9,18]. To limit the damage, research has been conducted with the aim of controlling the formation of ice crystals during the freezing of biological materials and foods [3,5,10,13,14,32,39]. The frequency of ice crystallization and the size of ice crystals depends on the latent heat discharge during the freezing process [33]. The time spent in the critical temperature zone (CTZ), leading to the release of latent heat, has been shown to be crucial to prevent the formation of ice crystals [9,28,37]. Indeed, the freezing of microscopic biological materials in liquid nitrogen can instantly vitrify their constituent elements thereby avoiding ice crystal formation [11,36]. However, a specific size of food (e.g. round fish or a chunk of meat) cannot be cooled uniformly, because all existing freezing systems draw heat from the surface. Therefore, rapid freezers with a high cooling performance, which minimizes the time that the target samples spend in the CTZ, have been commonly used for foodstuff freezing in the food industry despite their limitations [18].

A decade ago, the Cell Alive System® (CAS®), an additional device for the rapid freezer was produced by ABI Inc. (ABI Co., 2007). The device can generate weak oscillating magnetic fields (OMFs) in a rapid freezer. Despite the favorable reports of cryopreservation of small tissues using a programmed micro-freezer with CAS® in regenerative medicine [1,12,17,19,23,27,29,40,41] the inhomogeneous distribution of magnetic field intensity in the early models of large CAS® freezers showed little effect on food freezing [4,16,30,31,35,38]. Following improvements in the regulation of magnetic field conditions, the current model has been optimized for frozen food and has acquired international prominence in the food industry. Nonetheless, the effects of OMFs on the freezing of food products has remained an open question because of the general misunderstanding that the CAS® is an additional OMFs generator instead of a new freezing device [31] and the lack of analyses

* Corresponding author.
E-mail address: yomo@mukogawa-u.ac.jp (K. Yomogida).

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Table 1
Sample processing schedule.

Date of the received (= frozen date)	Number of mackerel received	Number of fillets	frozen fillets			Raw
			ABF	ABF-CAS	HF	
December 5, 2017	15	30	8	8	8	6
December 8, 2017	6	12	4	4	4	0
December 13, 2017	12	24	8	8	8	0
December 14, 2017	12	24	8	8	8	0
	total 45	total 90	total 28	total 28	total 28	total 6

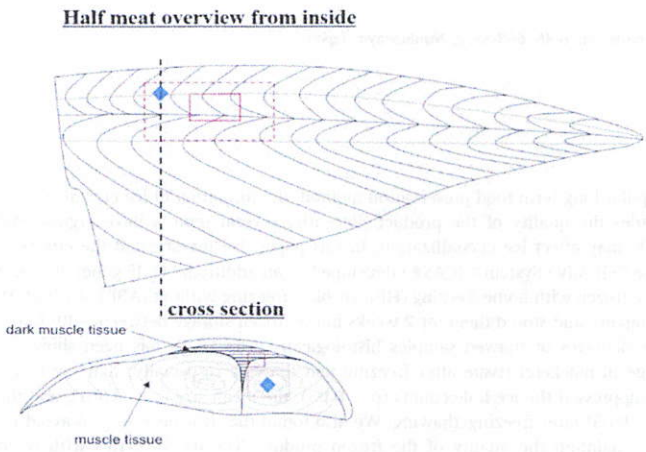

Fig. 1. Schema of a sample. The red frame and red dotted frame indicated portions isolated for histological analysis. Red dotted frame portion was isolated for chemical analyses. Blue diamonds indicate the temperature measurement positions. The samples used for temperature measurement were not used for other experiments.

considering the thawing process [4,30].

In this study, we selected mackerel (*Scomber japonicas*) as a target sample. Mackerel is not only a common fish in Japanese cuisine, but fresh mackerel is also ideal for eating raw in (e.g. in sushi or sashimi (sliced raw fish). However, it is hard to preserve the freshness of mackerel which negatively affects its value [7,8,25,26]. Moreover, long-term chilled storage of raw mackerel occasionally leads to proliferation of *Anisakis* [34] and the histidine to histamine conversion, both of which present a significant risk of food poisoning and other symptoms including urticaria or anaphylaxis [15]. Therefore, we expect that the establishment of an improved frozen storage method that maintains the quality of the fish may reduce these risks. To understand the thermodynamic effects of OMFs on the freezing of mackerel, we traced the center temperature change of samples during rapid freezing with and without OMFs, as well as during rapid thawing with an agitated mixture of ice and water (ice water) and the popular slow thawing method using a household refrigerator. To assess the practical effects of OMFs on food freezing, we analyzed the histological properties of the thawed sample after freezing with and without OMFs.

2. Materials and methods

2.1. Samples

After being caught at the south Sanriku coast in December 2017, 45 mackerels (*Scomber Japonicus*)(weight 790.2 ± 119.8 g, length 37.9 ± 2.4 cm; mean values ± SD) were immediately stored in ice and delivered

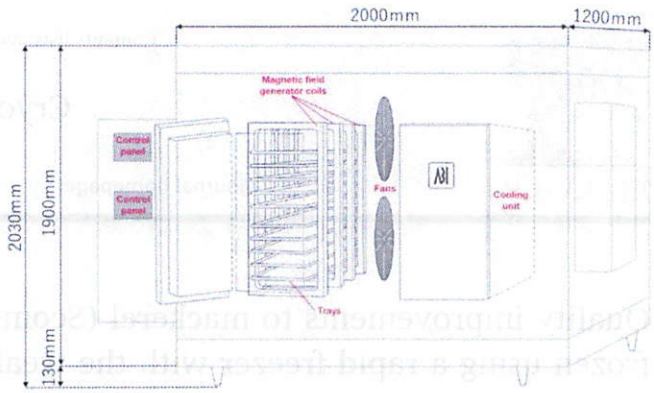

Fig. 2. Schematic drawing of the CAS freezer: a) Main components, b) Points at which magnetic field measurements were performed in freezing trays 1, 5, and 10.

to our laboratory within 24 h after capture using a refrigerated container service to ensure maximum freshness, also known as chub mackerel, Pacific mackerel, or Pacific chub mackerel (Table 1). As soon as the fish arrived, the heads and offal were removed and they were filleted (weight of each fillet 180.4 ± 33.3 g, thickness 20.6 ± 4.1 mm; mean values ± SD) for subsequent experiments (Fig. 1).

2.2. Freezing and frozen-storage conditions

Home freezing (HF): We arranged the samples in one layer on an aluminum tray in a household-style freezer set at −25 °C (model NR-C377M-P-, National Co., Ltd., Japan).

Air blast freezing with/without CAS® (ABF-CAS/ABF): An experimental batch air blast freezer, with and without CAS® was used. The freezer had been designed in consultation with ABI (F201003026, ABI Co., Ltd., Chiba, Japan) with the OMFs and control system supplied and commissioned by ABI. The basic construction of the freezer consisted of a cooling unit, a freezing cabinet, 2 fans, and 2 control panels. This cabinet contained a rack with 10 equidistant rails that would accommodate up to 10 aluminum trays for food freezing and the magnetic field generator. Different freezing conditions, namely air temperature (down to −50 °C), airflow (0–100%), and 'CAS energy' (0–700), could be set by the control panel (Fig. 2).

The freezer was previously cooled to −30 °C (precooling mode), and we placed the tray with samples arranged in one layer in the middle of the cabinet. After the sample was frozen, the air temperature was switched to −50 °C (rapid freezing mode) with the airflow 50% (approx. 0.5 m/s). With CAS® freezing, we set the CAS energy at 500, as recommended by ABI. Without CAS freezing, we turned off the CAS® energy. The OMF strength was measured using a gaussmeter (EMF-828, Fuso Co. Ltd., Tokyo, Japan). The OMF strength with CAS energy could be controlled in the range of 0.1–1.0 mT. The strength with CAS energy recommended for raw fish by ABI© was controlled at 0.1–0.5 mT. The freezing process was finished when the center temperature was −50 °C. After freezing, we immediately vacuum-sealed each sample in a polyethylene bag (vacuum pakkun super-roll®, Wide system Co., Ltd., Japan) with the unidirectional sheet (Fresh master, Unicharm Co., Tokyo, Japan) absorbing drip, and stored them for 2 weeks in the frozen storage (ABI Co. Ltd., Chiba, Japan) between −30 °C and −35 °C.

All the freezing experiments were performed in triplicate on separate days. Also, samples were randomly divided into three groups (HF, ABF-CAS, ABF).

2.3. Thawing conditions

Ice water thawing: Each sealed frozen sample was thawed in ice

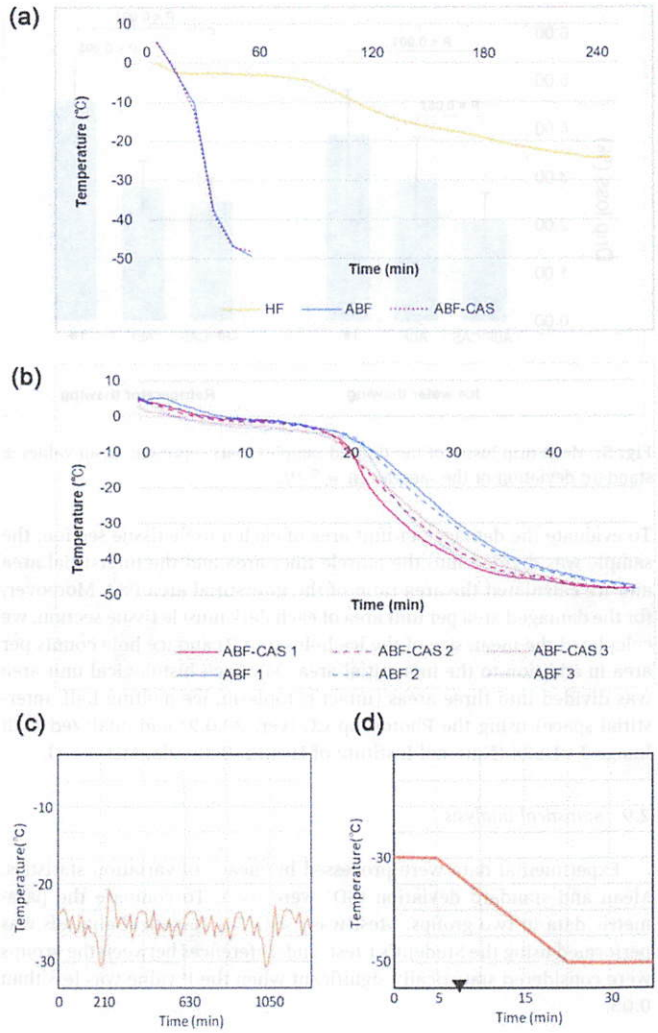

Fig. 3. Time-temperature curves during freezing processes.
(a) The mean center temperature changes in case of the household-style freezer, and the air blast freezer with or without CAS in the same time scale (n = 3 in each test). (b) Expanded time-temperature curves of each ABF/ABF-CAS sample in (a). (c) The inside temperature changes of the household-style freezer were monitored during the freezing process. (d) The inside temperature changes of the air blast freezer were monitored the freezing processes. Arrow head indicates the timing for placing sample in the freezing cabinet.

water until the center temperature was −0.5 to 0 °C [18]. The temperature of ice water was constantly monitored using a water-proofed thermocouple-K (0.32 mm diameter 1G, NND Co., Ltd., Japan). To uniform the ice water temperature, we continued agitation with several small submarine motors (ITEM70185, TAMIYA INC., Japan).

Refrigerator thawing: We placed each sealed frozen sample in the cold room of the refrigerator (model NR-C32Ep-H, National Co., Ltd., Japan) at 4 °C until the center temperature was −0.5 to 0 °C [18]. To confirm that each sample was completely thawed, we checked the hardness and measured the center temperature (testo 926-Starter set, Testo SE & Co. KGaA, Lenzkirch, Germany) before the analyses.

2.4. Temperature monitoring in real time

We monitored the center temperature of some samples (Fig. 1) during freezing and thawing using a thermocouple-type K (0.32 mm diameter 1G, NND Co., Ltd., Japan) connected to temperature recorder (MSR128-V6, M-System Co., Ltd., Japan) in real time. These samples were not used for further analyses. During sample freezing, the temperature inside of the freezer was also monitored in real time by a potable data logger (testo184T4, Testo SE & Co. KGaA, Lenzkirch, Germany).

2.5. Estimation of freezing

Since samples gradually freeze inwardly from the surface into the center, it is difficult to precisely determine the total freezing time. The center temperature is reflected outside of the latent heat due to ice crystallization during freezing. Consequently, we defined "the freezing time" as a time taken for the center of the sample to reach the setting temperature from the time that freezing was initiated, and "the time to freezing temperature" as a time taken from the center temperature reached to −20 °C from start temperature, because commercially food has been frozen down to −18 to −20 °C. Also, we defined the crystallization time as a time taken for the center temperature to pass through the CTZ (−1 °C to −7 °C) [21].

It has been suggested that the final quality of the frozen sample can be evaluated by the freezing rate. We calculated the freezing rate from the time when the center temperature reached −10 °C from −5 °C, as described [2,20,42].

2.6. Drip loss after thawing

Each sample was weighed using an electric balance (Max2100 g Mino.5 g e = 0.1 g d = 0.01 g, GX-2000, A&D Co., Ltd., Japan) before freezing. Each dry sheet (Fresh master, Unicharm Co., Tokyo, Japan) was weighed before and after use with an electrical balance (Max310 g Min0.02 g e = 0.01 g d = 0.001 g, GX-300, A&D Co., Ltd., Japan) Each drip loss was calculated as follows:

$$Drip\ loss(\%) = [(W_D - W_0)/W] \times 100 \quad (1)$$

where W_0 is the weight of the dry sheet before use (g), W_D the weight of the dripping sheet after use (g) and W the weight of the fresh sample (g).

2.7. Histological analysis

To analyze the histological change of samples due to freezing and thawing conditions, we cut off several small sections of tissue (5 × 5 × 5 mm) from the center of each sample (Fig. 1). The sections of each sample were fixed in Modified Davidson's solution (95% absolute ethanol, formalin, Acetic acid glacial and water = 33:22:11.5:33.5, also known as Hartmann's Solution) at 4 °C for 2 h and dehydrated in 70% ethanol at 4 °C overnight. Samples were subsequently dehydrated in different ethanol gradients (70% for 2 × 1 h, 80% and 95% for 1 × 1 h, 100% for 2 × 1 h) at 4 °C. The dried tissue samples were soaked in resin solution (Methyl methacrylate monomer, Dibutyl phthalate = 3:1, 75% benzoyl peroxide was added as a 34.3 mg/ml) at 4 °C overnight. We replaced resin solution with the fresh resin solution and added 1 vol% N, N-dimethylaniline to start the polymerization of the resin. Histological

Table 2
Estimation of freezing time.

	The freezing time	The time to freezing temperature (−20 °C)	Crystallization time	Freezing rate
	(min)	(min)	(min)	(°C/min)
HF	210.0 ± 0.0[a]	180.0 ± 28.3[a]	80.0 ± 0.0[a]	0.2 ± 0.0[a]
ABF	45.0 ± 0.0[b]	24.1 ± 1.6[b]	12.0 ± 2.4[b]	1.6 ± 0.4[b]
ABF-CAS	46.0 ± 0.0[b]	21.9 ± 0.9[b]	10.9 ± 1.9[b]	2.0 ± 0.9[b]

Data are expressed as mean ± SD. Different letters for mean values within a column represent significant different ($p < 0.05$).

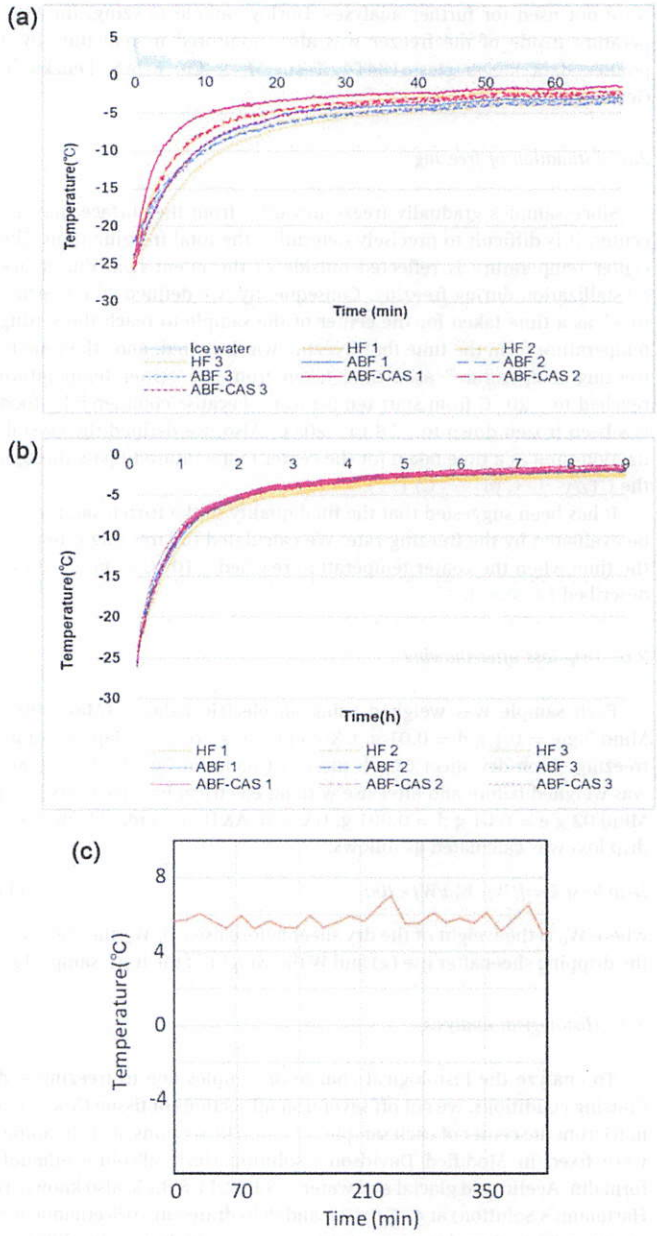

Fig. 4. Time-temperature curves during thawing processes. (a) The ice water and the sample center temperature changes during the ice water thawing (n = 3 in each test). (b) The center temperature changes during refrigeration (n = 3 in each test). (c) The inside temperature changes of home refrigerator monitored during the thawing process.

sections (thickness, 4 μm) of the resin-embedded tissues were prepared (RM2135, Leica Co., Ltd., Japan). The sections were stained with hematoxylin-eosin Stain and observed under a light microscope (BZ-800, KEYENCE Co., Japan).

2.8. Image analysis

To evaluate the damage per unit area of each muscle tissue cross-section, we applied the previous method with slight modifications. Two sections from each of the tissue samples per fish were used for the histological analyses of muscle tissue and dark muscle tissue from the various conditions. One to two spot areas from samples were randomly selected at low power and measurements performed at 10x (Muscle tissue) or 40x (Dark muscle tissue) magnification to avoid fatty tissue.

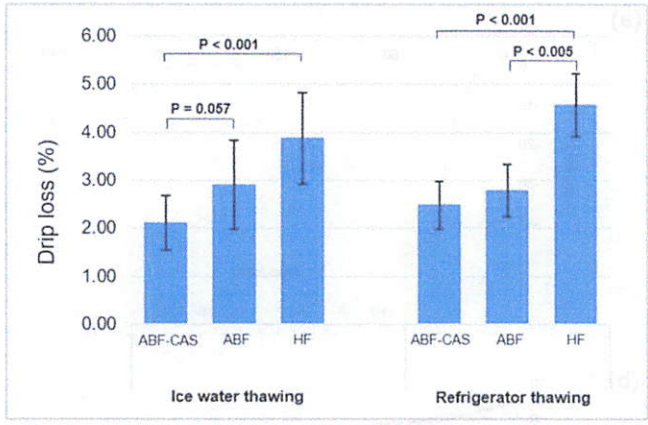

Fig. 5. Mean drip losses of the thawed samples. Bars represent mean values ± standard deviation of the samples (n = 5–9).

To evaluate the damage per unit area of each muscle tissue section, the sample was divided into the muscle fiber area and the interstitial area and we calculated the area ratio of the interstitial area (%). Moreover, for the damaged area per unit area of each dark muscle tissue section, we calculated the mean size of the ice holes (μm^2) and ice hole counts per area in addition to the interstitial area (%). Each histological unit area was divided into three areas (intact cytoplasm, ice melting hall, interstitial space) using the Photoshop CC (ver. 20.0.2) and analyzed with Image J v1.52a (National Institute of Health, Bethesda, MD, USA).

2.9. Statistical analysis

Experimental data were processed by means of variation statistics. Mean and standard deviation (SD) were used. To compare the parametric data in two groups, *t*-test was used. The statistical analysis was performed using the Student's t-test, and differences between the groups were considered statistically significant when the P value was less than 0.05.

3. Results

3.1. Freezing curve analysis

To understand the freezing performance of each freezer, we monitored the inside temperature of each device during the sample freezing (Fig. 3c and d). Since the home-freezer only had a weak device to circulate the air, the temperature in the freezer fluctuated between −20 °C and −30 °C (Fig. 3c). Conversely, the air blast freezer was able to control the inside temperature precisely during the freezing process (Fig. 3d) (see Table 2).

The mean freezing curves (n = 3) of the fillet during the HF, the ABF, and the ABF-CAS are shown in Fig. 3a. The crystallization time, the time to freezing temperature and the freezing time in HF were very extended and the freezing rate reflected the internal amount of ice crystals was only about 0.2 °C/min (Fig. 3a, Table 2). Although there was no significant difference between ABF and ABF-CAS in any endpoint ($p > 0.05$), "the time to freezing temperature" tended to shorten ABF-CAS than ABF ($p = 0.07$) (Fig. 3b, Table 2).

3.2. Thawing curves

The center temperature of the fillets was measured during thawing (Fig. 4). The thawing time in the refrigerator required five to six times as long as the thawing in ice water (Fig. 4a and b). Moreover, the thawing time in ice water was significantly different between ABF and ABF-CAS frozen samples ($p < 0.05$). The temperature in the refrigerator was kept

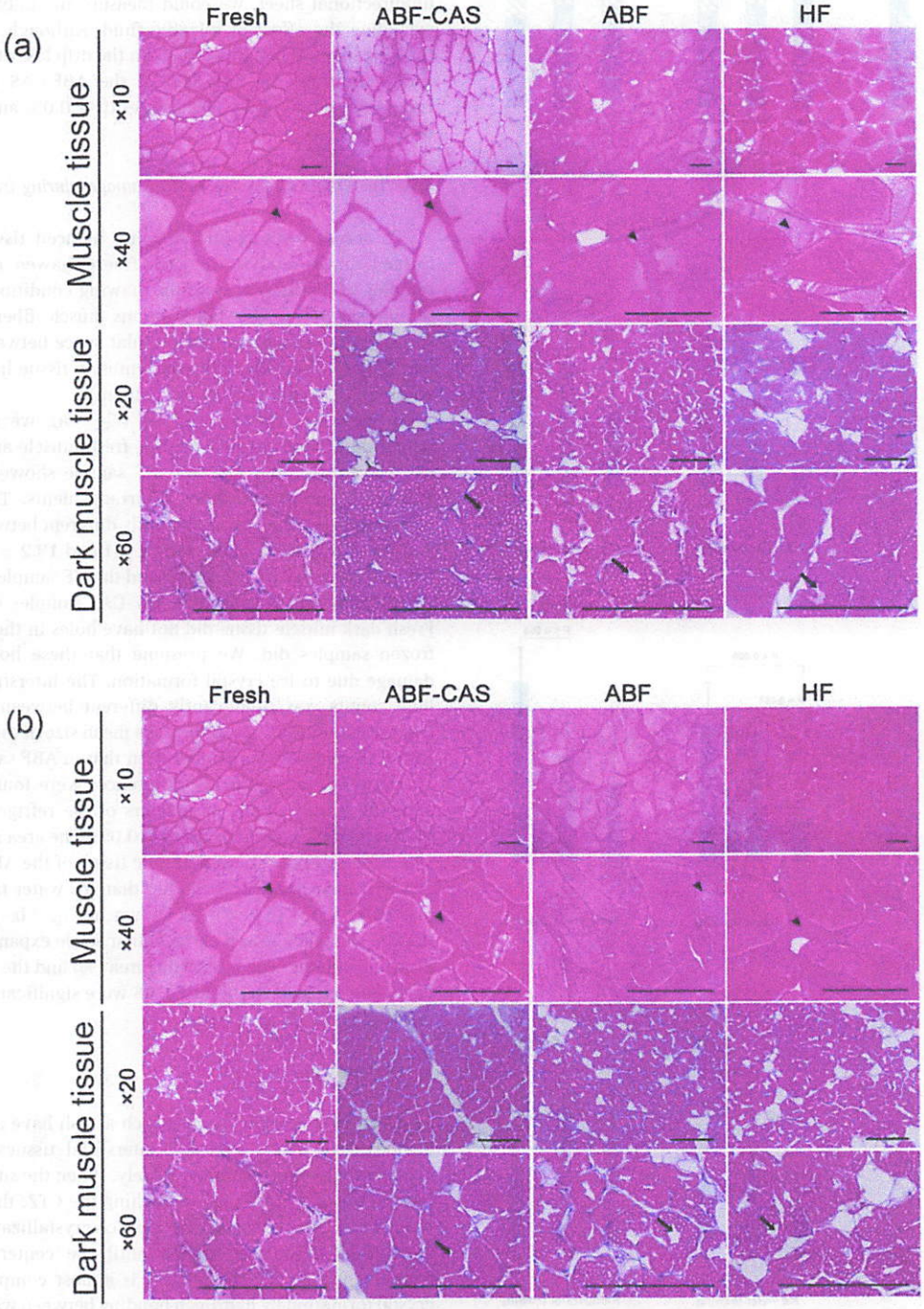

Fig. 6. Hematoxylin eosin stained histological cross sections of the muscle tissue and the dark muscle tissue. The fresh tissue and (a) the ice water thawing or (b) the refrigerator thawing tissues after 2 weeks of cryopreservation. Each arrow indicates ice crystal holes in the dark muscle fiber and each arrowhead indicates interstitial space in the muscle tissue. Each scale bar is 100 μm.

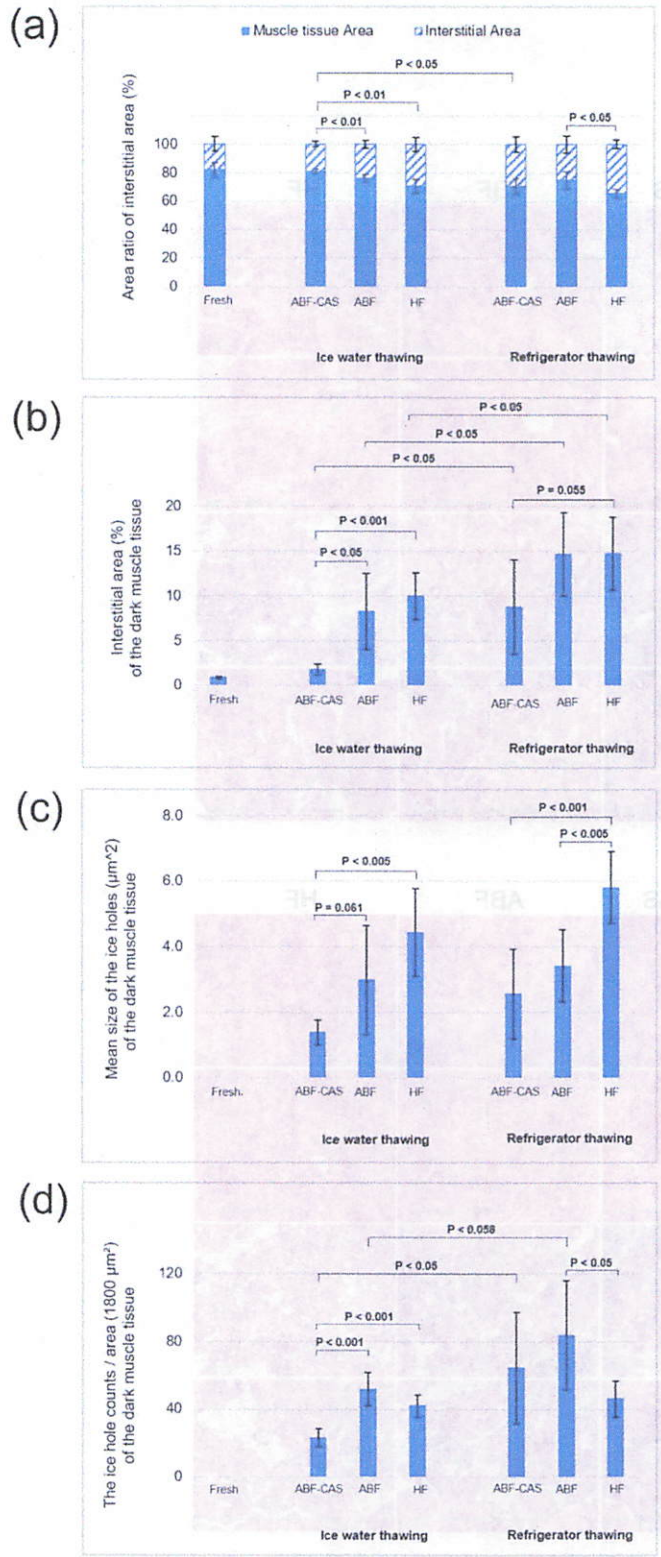

Fig. 7. Image analysis of the histology. (a) The ratio of muscle tissue area and the interstitial area in each muscle tissue (n = 5). (b) The ratio of the interstitial area in each dark muscle tissue (n = 6). (c) The mean size of the ice holes in each dark muscle tissue (n = 6). (d) The ice hole counts per 1800 μm2 in each dark muscle tissue (n = 6).

around 5 °C (Fig. 4c).

3.3. The drip loss of each thawed sample

The drip loss was evaluated immediately after the thawing process to avoid any additional reactions or modifications in volume. Using the unidirectional sheet, we could measure the only drip by freeze-thaw excluding the effect of relaxing fluid. Although the difference in the thawing method had little effect on the drip loss of the sample frozen by each condition, the drip loss of the ABF-CAS sample was reduced compared to that of the HF samples ($p < 0.05$) and to that of ABF ($p = 0.057$) (Fig. 5).

3.4. The OMFs suppressed tissue damages during freezing/thawing

To access freezing and thawing induced tissue damage, we performed image analysis of each freeze-thawed mackerel histological samples with different freezing/thawing conditions (Fig. 6). The fresh sample showed a very homogenous muscle fiber distribution, no fissures, with very narrow extracellular space between the muscle tissue, and the cross-section of the dark muscle tissue had relatively uniform and regular shapes (Fig. 6, left column).

In ice water thawed samples (Fig. 6a), we observed no obvious structural differences between the fresh muscle and the ABF-CAS muscle. In contrast, the ABF and HF sample showed extracellular space expansion and muscle fiber disarrangements. The area ratio of the interstitial area was not significantly different between the fresh samples and the ABF-CAS samples (18.3 ± 5.1 and 19.2 ± 1.8%, $p < 0.05$), but the ABF samples (24.0 ± 2.6%) and the HF samples (29.5 ± 4.8%) were significantly higher than the ABF-CAS samples ($p < 0.01$) (Fig. 7a). Fresh dark muscle tissue did not have holes in the muscle fibers, while frozen samples did. We presume that these holes are the result of damage due to ice crystal formation. The interstitial area (%) and ice hole counts was significantly different between ABF-CAS and other freezing conditions ($p < 0.05$). The mean size of the ice holes (μm) in the ABF-CAS samples was smaller than that in ABF samples ($p = 0.061$).

However, no significant differences were found between ABF-CAS and ABF in any of the parameters of the refrigerator thawed muscle tissue and dark muscle tissue ($p > 0.05$). The area ratio of the interstitial area of refrigerator thawed muscle tissue of the ABF-CAS samples (29.2 ± 5.5%) was significantly higher than ice water thawed ABF-CAS samples ($p < 0.05$) (Fig. 7). As shown in Fig. 6b, ABF-CAS refrigerator thawed samples showed extracellular space expansion and muscle fiber disarrangements. The interstitial area (%) and the ice hole counts of the dark muscle tissue in the ABF-CAS were significantly different between thawing methods ($p < 0.05$).

4. Discussion

Since fresh freezing targets such as fish have a specific size and are composed of many cells and interstitial tissues, it is impossible to completely freeze them immediately. When the surface layer of a target sample starts to freeze upon reaching the CTZ, the center temperature must be in the plateau phase (the ice crystallization time) due to the latent heat from the outside until the center portion has frozen completely (Fig. 8). Latent heat is almost completely due to the ice crystal formation by hydrogen bonding between water molecules. Latent heat might not only prevent freezing of the inside layer, but also extends the crystallization time. Indeed, the temperatures of freezing plataue were different between HF and ABF/ABF-CAS (Fig. 3, Table 2).

In this study, we examined the effect of CAS® on freezing and the effect of different thawing methods on ABF-CAS frozen samples. Since there are variations in each fish fillet size, uniform gel models were often used for many freezing temperature-time curve analyses [10,30]. However, the uniform gel model could not accurately reproduce the temperature change of real fish with complex internal tissue structures

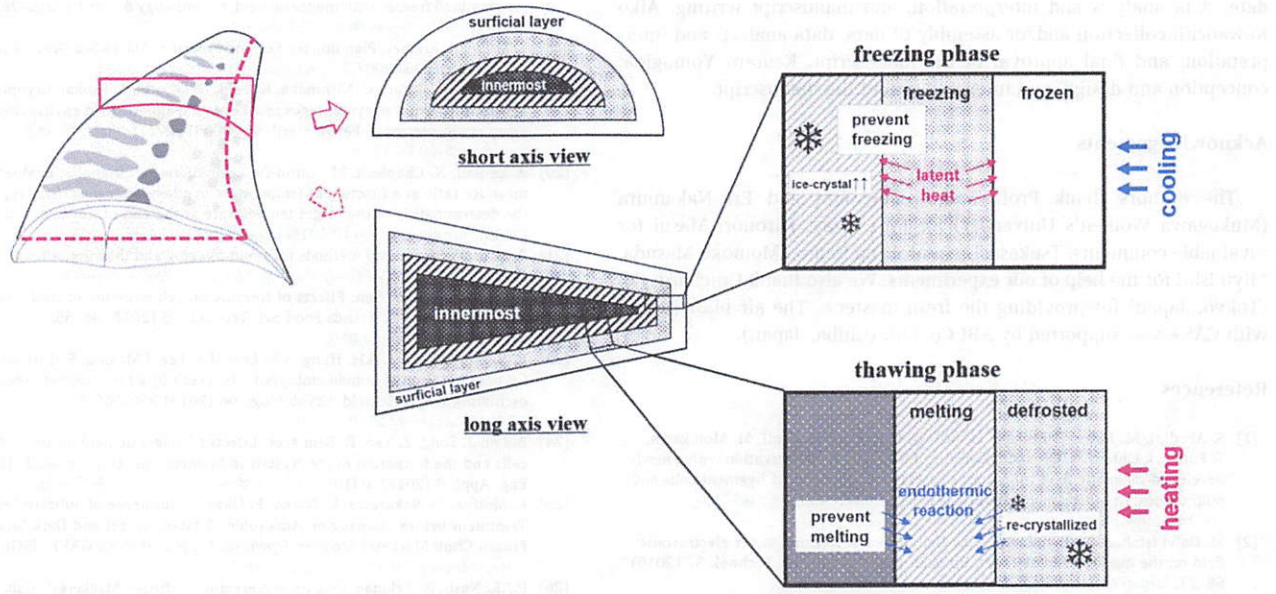

Fig. 8. Hypothesis of the heat transfer in a certain size of fish meat during freezing and thawing.

[22]. The mackerel fillet used this time was difficult to accurately analyze the freezing temperature curve due to the variation in shape. Therefore, we have confirmed that the use of uniformly sized tuna sections significantly shortened the "the time to freezing temperature" ($p < 0.05$) and tended to shorten the crystallization time ($p = 0.084$) by OMFs (Data in Brief).

Our data suggests that the OMFs inhibit latent heat by suppressing the hydrogen bonding between water molecules during ABF, as previously reported [24]. Indeed, we could only detect very slight tissue damage due to the ice crystal formation in the ABF-CAS samples (Figs. 6 and 7). In ice water thawed samples (Fig. 6a), we observed no obvious structural differences between the fresh muscle and the ABF-CAS muscle. Moreover, in the dark muscle tissue, the interstitial area (%) and ice hole counts were significantly different between ABF-CAS and other freezing conditions ($p < 0.05$). These results suggest that the OMFs has been inhibited histological damage in mackerel tissue after freezing and thawing (especially, thawing in ice water). Therefore, the OMFs could be suppressed to ice crystal formation in the rapid freezing process. Alternatively, the OMFs may promote that the conductivity was improved or that the OMF enhanced crystallization throughout the product.

The tissue of vertebrate such as a fish is composed of many cells and interstitial space. Expansion of the content volume due to the ice crystallization in the space surrounded by biological membranes leads to breaks in the membrane structure allowing biological content to spread into the outer space. This damage increases as crystallization time increases. Such histological damage in a certain wide tissue area should be scanned with the light microscope and be evaluated quantitively (Figs. 6 and 7). Although an electron microscope is a useful tool to investigate micro-structure damage, it is not suitable to quantify a certain size of tissue damage for the extreme micro-field of view.

During the thawing process, an endothermic reaction should occur to break off the hydrogen bond in ice crystals. If the heat exchange efficiency is low, the freshly thawed layer could be occasionally re-crystallization by the endothermic reaction from the inner layer (Fig. 8). Our data suggested that inadequate slow thawing increases the probability of repeated re-crystallization and re-thawing of samples (Fig. 4b). The severe damage of refrigerator thawed tissues could be due to the repeating ice crystallization (Figs. 6–7). Unfortunately, various thawing methods with insufficient consideration drew the different views on the freezing effects of OMFs in previous researches [4,30]. To prevent such tissue damage, an effective thawing method like the ice water thawing method used in this paper should be selected [6]. Interestingly, the ice water thawing time of the ABF-CAS sample seemed to be shortened than that of the ABF sample. The ABF-CAS sample might require less endotherm to melt ice crystals owing to the suppression of ice crystal formation by OMFs. The monitoring temperature valiations for each thawing plataue should be due to the individual sample difference. As mentioned in methods, we confiermed directly that each center temperature was −0.5 to 0 °C with another thermometer.

Although the global Japanese food boom affects the value of fresh fish which is intended to be eaten raw, it has been impossible to maintain freshness for a long period of time without the necessary improvements in freezing technologies. As we have described above, the optimized OMFs provided by CAS® has the potential to maintain the quality of frozen fresh foods. The prevention of tissue damage due to ice crystallization provides high quality frozen fresh food in food engineering as well as excellent viability of cells/organs in bioscience.

5. Conclusion

The OMFs has been shown to inhibit histological damage in mackerel tissue after freezing and thawing (especially, thawing in ice water). And it suggests that OMFs significantly suppressed the ice hole counts ($p < 0.05$) and increase of interstitial area% ($p < 0.05$) after freezing/thawing. Therefore, the OMFs could be suppressed to ice crystal formation in the freezing process. Alternatively, the OMFs may promote that the conductivity was improved or that the OMF enhanced crystallization throughout the product.

Since tissue damage by ice crystallization could be detected very slightly in the ABF-CAS samples, the OMFs might suppress the latent heat generation by inhibiting the hydrogen bonding between water molecules. However, to evaluate the effect accurately, it is necessary to select an appropriate thawing method that prevents secondary ice crystal damage during thawing. The benefits of the application of the OMFs could be two-fold; fresh quality frozen foods for the food industry and, increased viability of frozen stock cells/organs for bioscience/medicine.

Author contributions

Kana Okuda: conception and design, collection and/or assembly of

date, data analysis and interpretation, and manuscript writing. Aiko Kawauchi: collection and/or assembly of data, data analysis and interpretation, and final approval of the manuscript. Kentaro Yomogida: conception and design, and interpretation of the manuscript.

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