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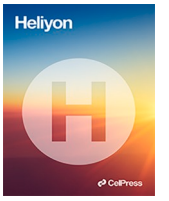

Research article

Changes in beef protein digestibility in an *in vitro* infant digestion model with prefreezing temperatures and aging periods

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ABSTRACT

The protein digestibility of beef at three prefreezing temperatures (freezing at −20 °C, F20; freezing at −50 °C, F50; and freezing at −70 °C, F70) and aging periods (4, 14, and 28 days) was investigated using an *in vitro* infant digestion model. The increased cathepsin B activity in the frozen-then-aged treatments ($P < 0.05$) resulted in a higher content of 10% trichloroacetic acid-soluble α-amino groups than in the aged-only group on days 14 and 28 ($P < 0.05$). F50 had the most α-amino groups in the digesta and digested proteins under 3 kDa on day 28 ($P < 0.05$), with the disappearance of actin band in the digesta electrophoretogram. The secondary and tertiary structures of myofibrillar proteins revealed that F50 underwent irreversible denaturation ($P < 0.05$), especially in the myosin fraction, while F20 and F70 showed protein renaturation during aging ($P < 0.05$). In general, prefreezing at −50 °C then aging can improve the *in vitro* protein digestibility of beef through freezing-induced structural changes.

1. Introduction

Meat is considered an excellent source of baby food because it contains high-quality proteins with balanced amino acid compositions and has high digestion and absorption rates. Because protein plays a direct role in postprandial muscle anabolism, the intake of high-quality protein is important for infants, whose protein requirement are higher than that of young adults [1]. Since infants require a large amount of nutrients for growth and development between 6 and 24 months of age, they must be fed complementary foods, such as proteins, to meet the increased nutrient requirement. Therefore, the World Health Organization [2] recommends serving meat as a solid or semi-solid food to infants 6–24 months of age daily. Although meat proteins are highly digestible, infants have immature digestive systems and cannot adequately digest meat proteins due to the low secretion of digestive enzymes and fluids [3]. Since this can lower the nutritional quality of dietary proteins by causing several health problems (diarrhea, allergic reactions, and reduced growth rate), improving the digestibility of meat protein can improve the nutritional quality of meat and have several positive effects on infant health.

Freezing is a conventional technology used in the meat industry to extend the shelf life of meat and meat products. Freezing meat drastically changes the environment that surrounds the proteins and influences their stability. The disruption of muscle cell structure by the formation of ice crystals, cold denaturation due to reduction of the free energy barrier for protein unfolding, and freeze-concentration modifying pH and accelerating protein oxidation are three factors that affect protein stability during freezing [4,5].

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The main factors causing these changes is the freezing rate and cold denaturation can occur differently depending on the freezing temperature [5,6]. Therefore, the difference in ice crystal formation and the occurrence of cold denaturation depending on the freezing rate can affect the modification of the surrounding proteins. Freezing can affect the degree of proteolysis during storage because it also influences the activities of endogenous proteases in meat as the physicochemical changes in muscle during freezing storage can also affect the activities of the proteases such as calpain and cathepsin during aging [7]. These events not only affect the functional and textural characteristics of thawed meat *via* structural changes in proteins, but also control the nutritional quality of proteins by changing the accessibility of digestive enzymes [8].

We hypothesized that, depending on the freezing rate, changes in the protein structure during subsequent aging can affect the substrate accessibility of digestive proteases. Although the original protein structure is better maintained when small ice crystals are formed rather than large crystals [9], physicochemical structural changes (disruption of muscle fibers and unfolding of proteins) can enhance muscle protein degradation, resulting in a potential increase in the digestive availability of proteins due to the exposure of buried specific substrates to digestive enzymes [6]. Therefore, in this study, we investigated how aging beef after freezing pretreatment at various temperatures (−20, −50, and −70 °C) affected endogenous protease activity, the secondary and tertiary structures of myofibrillar proteins, and proteolysis during aging. The appropriate conditions for improving protein digestibility were also investigated using an *in vitro* model of the infant digestive tract.

2. Materials and methods

2.1. Sample preparation

Six (semitendinosus) muscles from three heifer carcasses were purchased 24 h postmortem from a local market (Daejeon, Korea). The excessive fats and connective tissues were removed before the sample allocation. Two muscles from the same carcass were divided into 13 pieces (2.5 cm thick) and randomly allocated to day 0 (fresh samples), four treatments (AO, aging only; F20, F50, and F70, frozen at −20, −50, and −70 °C, respectively), and three storage periods (4, 14, and 28 days). Two muscles obtained from the same carcass were considered as one batch, and a total of three batches were used (replications). The vacuum-packaged samples were frozen at −20, −50, and −70 °C for 48 h, thawed at 4 °C for 48 h (referred to as the freeze-thawed samples that were not additionally aged), and subsequently aged at 4 °C for 14 and 28 days. The AO samples were aged under the same conditions without freezing. All three batches were subjected to freezing and aging on the same day and the samples were collected and stored at −70 °C for analysis after pH monitoring after 14 or 28 days.

2.2. Calpain and cathepsin B activities

Calpain activity was monitored using an assay kit (Abcam, ab65308; Cambridge, Massachusetts, USA), according to the protocol provided by the manufacturer. The fluorometric assay was based on the detection of a cleaved calpain substrate (Ac−LLY−AFC). Fluorescence intensity was measured at excitation and emission wavelengths of 400 and 505 nm, respectively (Thermo-Fisher Scientific, Varioskan LUX, MA, USA).

Cathepsin B activity was detected using a cathepsin B substrate (Sigma, Z-Arg-Arg-AMC, St. Louis, MO, USA) according to the method described by Lomiwes et al. [7]. Fluorescence intensity was recorded using a plate reader (Varioskan LUX) at excitation and emission wavelengths of 355 and 460 nm, respectively.

Both calpain and cathepsin B activities were expressed as fold increases compared to that of fresh beef (day 0).

2.3. α-Amino group contents

The samples were reacted with *o*-phthaldialdehyde (OPA) to quantify α-amino groups in the sample filtrate and digesta according to the method previously reported by Lee et al. [10]. The absorbance of the reaction mixture was measured at 340 nm using a plate reader (Varioskan LUX). Glycine was used to prepare the standard curve and the Kjeldahl method (AOAC method 928.08) [11] was used for crude protein analysis.

2.4. Protein carbonyl contents

Protein carbonyl content was used to monitor protein oxidation. The carbonyl content was measured according to the method previously reported by Estévez [12], using samples extracted in sodium phosphate buffer with dinitrophenylhydrazine. The absorbance of the reaction mixture was measured at 370 nm using a plate reader (Varioskan LUX). The protein content was determined using a Bio-Rad protein assay (#5000006). The protein carbonyl content was expressed as nmol carbonyl/mg protein using a molar absorptivity of 22,000 M^{-1} cm^{-1}.

2.5. Myofibrillar protein preparation

Myofibrillar proteins were extracted according to the method described by Lee et al. [10]. The final pellet sample was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) and homogenized to form a myofibrillar protein extract. The protein content was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, #5000006, Richmond, CA, USA), and bovine serum albumin was used

to generate a standard curve.

2.6. Endogenous tryptophan fluorescence intensity

The fluorescence intensity of tryptophan was determined according to the method described by Qian et al. [13]. The myofibrillar protein suspensions were diluted to a concentration of 0.5 mg/mL with 0.6 M NaCl dissolved in 20 mM potassium phosphate buffer (pH 6.0). The fluorescence spectra were recorded between 300 and 400 nm at an excitation wavelength of 280 nm using a plate reader (Varioskan LUX).

2.7. Surface hydrophobicity

The surface hydrophobicities of the samples were monitored using the bound bromophenol blue (BPB) content, as described by Chelh et al. [14]. The myofibrillar protein suspension (protein concentration, 1 mg/mL) was reacted with BPB then centrifuged. The supernatant was diluted 10-fold and the absorbance was measured at 595 nm (Varioskan LUX). A control sample was prepared by reacting the phosphate buffer with BPB. The bound BPB content was expressed by the following equation:

$$\text{Bound BPB } (\mu g) = 200\ \mu g \times (\text{absorbace of control} - \text{absorbance of sample}) \div \text{absorbance of control}$$

2.8. Preparation of myosin and actin fractions

Beef samples (1 g) were homogenized (IKA GmbH & Co. KG, T25 basic, Staufen Germany) with 10 mL of isolation buffer (1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 M KCl, 2 mM $MgCl_2$, and 10 mM potassium phosphate (pH 7.0)), then centrifuged at 2000×*g* for 10 min (LABOGENE Co., Ltd., 1580R, Lynge, Denmark). Then, 5 mL of Tris buffer containing 5 mM Tris, 1 mM 1,4-dithiothreitol, and 1 mM EGTA (pH 7.8) was added to the pellet, and the mixture was homogenized and centrifuged again. The pellet was then stirred with an aliquot (5 mL) of 150 mM potassium phosphate buffer (pH 6.5) containing 150 mM ethylenediaminetetraacetic acid for 20 min then centrifuged. G-S buffer (5 mM $MgCl_2$, 0.4 M NaCl, 150 mM Na_3PO_4, and 5 mM $Na_4P_2O_7$, pH 6.0) was added to the pellet, and the mixture was stirred for 20 min and centrifuged. The supernatant (A) and pellet (B) were collected for myosin and actin fractions, respectively. For the myosin fraction, an equal volume of distilled water was added to supernatant A, and the mixture was centrifuged. The pellet was used for the myosin fraction after homogenization with 0.1 M potassium phosphate buffer (pH 7.8). For the actin fraction, the pellet (B) was washed two more times with G-S buffer, and the final pellet was mixed with 0.1 M potassium phosphate buffer (pH 7.8). The mixture was used as the actin fraction. The SDS-PAGE electrophoretogram of the myosin and actin fractions are shown in Supplementary Fig. 1.

2.9. Circular dichroism (CD) spectroscopy

The secondary structures of the myosin and actin fractions were monitored by CD spectroscopy (Applied Photophysics, Chirascan VX, Leatherhead, UK). The extracts were diluted to a protein concentration of 0.5 mg/mL using the Qubit® Protein Assay Kit (ThermoFisher Scientific, A50669, Massachusetts, USA). The response time, scan rate, and bandwidth were 0.25 s, 100 nm/min, and 1.0 mm, respectively. Two replicates were used to obtain a single spectrum. CD data were expressed in millidegrees, and the scanning range was between 200 and 260 nm. CDNN software (version 4.0) was used to estimate the relative contents of the secondary structural components (α-helix, β-sheet, β-turn, and random coil) in the obtained spectrum.

2.10. In vitro digestion

Distilled water was used to dilute the ground beef samples to the same protein content to prepare beef puree (50 mg protein/mL beef puree). The mixture was cooked at 80 °C until reaching the core temperature of 75 °C, cooled to 25 °C, and homogenized at 12,000 rpm for 30 s (T25 basic).

The *in vitro* digestion model simulated the digestive tract of infants six months after birth based on previous studies. To simulate the infant digestive tract, an electrolyte stock solution was prepared according to the studies of Passannanti et al. [15] and Bourlieu et al. [3]. The simulated gastric fluid (pH 3.8) contained 1000 U/mL pepsin from porcine mucosa and 21 U/mL gastric lipase from *Rhizopus oryzae*, whereas the simulated intestinal fluid (pH 7.0) contained 100 U/mL trypsin and 15 U/mL chymotrypsin from bovine pancreas, and 200 U/mL pancreatic lipase and 4 mM bile extract from porcine pancreas.

As suggested by Minekus et al. [16], who studied the INFOGEST *in vitro* digestion model, the digestive fluid in each compartment mixed with the fluid from the previous compartment in a 50:50 (v/v) ratio during digestion. The beef puree (10 mL) was added to an equal volume of gastric fluid and the mixture was digested at 37 °C for 2 h. Subsequently, 20 mL of simulated intestinal fluid was added and mixed again at 37 °C for 2 h. Control samples were digested under the same conditions by adding distilled water instead of the beef puree samples to exclude the protein content of digestive fluids. Gastrointestinal digesta samples were immediately stored at −70 °C until analysis.

2.11. Content of digested proteins under 3 kDa

The digesta samples were size fractioned using centrifugal filters (Millipore, Amicon Ultra-15, Billerica, MA, USA) according to the protocol provided by the manufacturer. The digesta samples were filtered through a centrifugal filter with a 10 kDa molecular weight cut-off (MWCO), and the filtrate was centrifuged again and passed through a filter with a MWCO of 3 kDa. The protein content of the final filtrate was measured using the Kjeldahl method (AOAC method 928.08) [11]. Protein digestibility after *in vitro* digestion was calculated using the following equation:

$$\text{Digestibility } (\%) = 100 \times (\text{protein content in the filtrate} - \text{protein content of the control filtrate}) \div \text{protein content in the digesta}$$

2.12. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The digesta samples (1 mg/mL) were diluted with 2X sample buffer (EBA-1051, Elpis Biotech, Daejeon, Korea) at a 1:1 (v/v) ratio and heated at 90 °C for 10 min in a heating block. Electrophoretic separation was conducted using 12.5% polyacrylamide gels. The protein bands were stained with Coomassie brillant blue buffer overnight and destained with acetic acid buffer. The gel was scanned using Epson Perfection V850 Pro (Epson, CA, USA).

2.13. Statistical analysis

This study used three iterations with three batches, and all the results were analyzed using a mixed model with a randomized complete block design. Fixed effects for the results included the prefreezing temperatures (−20, −50, and −70 °C) and aging periods (0, 4, 14, and 28 days) and the batches (carcasses) were described as random effects. The results were expressed as the least squares mean and standard error of the least squares means. Tukey's multiple comparison test was used to evaluate the significance of the main effects ($P < 0.05$). The SAS program (version 9.3, SAS Institute Inc., Cary, NC, USA) was used to perform statistical analyses.

3. Results and discussion

The central concept of this study was to increase the digestive availability of beef proteins by freezing-then-aging treatment. We employed three prefreezing temperatures of −20, −50, and −70 °C. The glass transition temperature of beef has been reported to be between −12 and −20 °C [9], so we used −20 °C as a slow-freezing temperature. Moreover, as Arsiccio et al. [5] observed the cold denaturation of proteins at 230 K (−43.15), the −50 and −70 °C were used to induce the cold denaturation. As three freezing temperatures can induce the physicochemical changes in beef proteins by different mechanisms, we assumed that difference in the prefreezing temperature would result in the different proteolytic behaviors of beef during aging and *in vitro* protein digestion herein.

Table 1
Activities of calpain and cathepsin B (fold-increase compared to 0 day) and the contents of 10% TCA-soluble α-amino groups (mM/g) of beef samples during aging for 28 days.

	Aging periods (day)				
Treatment	0	4	14	28	SEM[a]
	Calpain activity				
AO	1^{a}	$0.68^{B,b}$	0.66^{b}	$0.67^{A,b}$	0.015
F20	1^{a}	$0.86^{A,b}$	0.63^{c}	$0.60^{AB,c}$	0.016
F50	1^{a}	$0.85^{A,b}$	0.69^{c}	$0.56^{AB,c}$	0.049
F70	1^{a}	$0.75^{AB,b}$	0.69^{b}	$0.53^{B,c}$	0.018
SEM[a]	–	0.036	0.033	0.031	
	Cathepsin B activity				
AO	1^{c}	$0.97^{B,c}$	$1.15^{C,b}$	$1.32^{B,a}$	0.022
F20	1^{c}	$1.02^{AB,c}$	$1.33^{B,b}$	$1.46^{A,a}$	0.018
F50	1^{b}	$1.11^{A,b}$	$1.56^{A,a}$	$1.48^{A,a}$	0.034
F70	1^{b}	$1.01^{AB,b}$	$1.43^{AB,a}$	$1.48^{A,a}$	0.022
SEM[a]	–	0.031	0.049	0.024	
	10% TCA-soluble α-amino groups				
AO	0.14^{c}	0.15^{b}	$0.16^{B,b}$	$0.21^{B,a}$	0.003
F20	0.14^{d}	0.15^{c}	$0.19^{A,b}$	$0.27^{A,a}$	0.002
F50	0.14^{d}	0.15^{c}	$0.20^{A,b}$	$0.27^{A,a}$	0.002
F70	0.14^{d}	0.15^{c}	$0.20^{A,b}$	$0.26^{A,a}$	0.002
SEM[a]	0.002	0.001	0.002	0.003	

A-C Different uppercase letters indicate significant differences in mean between treatments in the same aging period ($P < 0.05$).
a-d Different lowercase letters indicate significant differences in mean between aging periods in the same treatment ($P < 0.05$).
[a] Standard error of the least square mean.

3.1. The activities of endogenous proteases and protein degradations in beef

Postmortem proteolysis of cytoskeletal and myofibrillar proteins is believed to be predominantly due to the activities of calpains and cathepsins. Calpain, a calcium-dependent protease, is activated after slaughter by the release of calcium ions from the sarcoplasm reticulum into the sarcoplasm [7]. In this study, calpain activity in beef was highest on day 0 and decreased with aging (Table 1, $P < 0.05$). However, freezing pretreatment at −20 and −50 °C (F20 and F50) showed significantly higher calpain activity on day 4 than AO ($P < 0.05$). There were no significant differences in calpain activity between AO, F20, and F50 during subsequent aging periods. Freezing beef at −70 °C (F70) before aging had no effect on calpain activity throughout the aging period compared to AO ($P > 0.05$). Moreover, F70 had the lowest calpain activity of the four treated samples on day 28 ($P < 0.05$, although there was no significant difference between the frozen-then-aged samples). The decrease in calpain activity during aging is related to the autolysis of calpain and activation of calpastatin, which is calcium-dependent and antagonistic to calpain activity [7]. In this study, beef pretreated by freezing for 48 h and then thawing for 48 h was aged for 4 days. Therefore, the decrease in calpain activity in frozen-then-aged beef may be delayed during storage at low temperatures. Moreover, previous studies have reported the inhibition of calpastatin activity after freezing meat at −20 °C [17] and −30 °C [18], while Kristensen et al. [19] reported constant calpastatin activity after fast freezing at −80 °C. Therefore, F70 seemed to have calpain activity similar to that of AO during aging.

Regardless of freezing temperature, cathepsin B activity in beef significantly increased with the frozen-then-aged treatments on days 14 and 28, compared with AO. Cathepsin is in the lysosome, where it has no proteolytic activity on muscle proteins prior to slaughter. Damage to the lysosome results in the release of cathepsin into the sarcoplasm, which can break down meat proteins [20]. Under freezing conditions, ice crystals formed *via* the aggregation of water molecules weaken muscle structure and damage muscle organelles, including lysosomes [9]. Therefore, the results of this study demonstrated that freezing damaged lysosomes and increased the proteolytic activity of cathepsin during subsequent cold storage. Meanwhile, F50 showed the highest cathepsin B activity on days 4 and 14 among the treatments ($P < 0.05$). Therefore, it appears that, of the three freezing temperatures, freezing beef at −50 °C is the most appropriate condition to activate cathepsin for postmortem proteolysis. This is further discussed along with the results of the structural changes in Section 3.3.

It should be noted that cathepsin activity increased from day 0 to day 28 of aging (Table 1, $P < 0.05$), while calpain activity gradually decreased. Both calpain and cathepsin can degrade lysosomes [21,22]. At the beginning of aging, cathepsin could have been released by the disruption of lysosomes due to the decrease in pH, and then, during the late stage of aging, released after the degradation of lysosomes by endogenous proteases. Moreover, cathepsin and calpain have optimal activities under acidic and neutral conditions, respectively [22,23]. Since the beef samples used in this study had a low ultimate pH of 5.39 at day 0 (data not shown), cathepsins might have more suitable conditions to retain their activities than calpains.

We monitored the content of 10% TCA-soluble α-amino groups to estimate the degree of proteolysis during aging because low-molecular-weight peptides and free amino acids with 3–4 residues are solubilized in 10% TCA solution [10]. The frozen-then-aged beef had a higher α-amino group content than AO beef from days 14–28 ($P < 0.05$, Table 1). These results indicate that postmortem proteolysis occurred more frequently in the frozen-then-aged treatments than in AO treatment, which was caused by an increase in the activity of endogenous proteases, especially cathepsin (Table 1). Although F50 temporarily showed significantly higher cathepsin B activity than F20 on day 14, no differences in the activities of the frozen-then-aged treatments at days 4 and 28 could have resulted in similar levels of 10% TCA-soluble α-amino groups.

3.2. Protein oxidation in beef

The protein carbonyl group content was measured to monitor the occurrence of protein oxidation after freezing and aging (Table 2). F20 had a higher carbonyl content than AO on day 4 ($P < 0.05$), although there was no significant difference between AO and the other frozen-then-aged treatments. This can be attributed to the higher oxidative environment in F20 than in any other treatment due to the solute concentration during slow freezing [4].

There was no significant difference in carbonyl content among the treatments on days 14 and 28 of aging (Table 2). Interestingly, peptides derived from the degradation of meat proteins during postmortem proteolysis have been reported to have various biological activities, including antioxidant activity [24]. In this study, the frozen-then-aged treatments had higher 10% TCA-soluble α-amino

Table 2
Contents of protein carbonyl (nmol/mg) of beef samples during aging for 28 days.

	Aging periods (day)				
Treatment	0	4	14	28	SEM[a]
	Protein carbonyl				
AO	2.74^{b}	2.46^{B,b}	2.55^{b}	3.76^{a}	0.166
F20	2.74^{b}	2.92^{A,b}	2.84^{b}	3.62^{a}	0.097
F50	2.74^{b}	2.70^{AB,b}	2.92^{b}	3.35^{a}	0.121
F70	2.74^{b}	2.72^{AB,b}	2.68^{b}	3.32^{a}	0.114
SEM[a]	0.087	0.131	0.091	0.227	

$^{A-B}$ Different uppercase letters indicate significant differences in mean between treatments in the same aging period ($P < 0.05$).
$^{a-b}$ Different lowercase letters indicate significant differences in mean between aging periods in the same treatment ($P < 0.05$).
[a] Standard error of the least square mean.

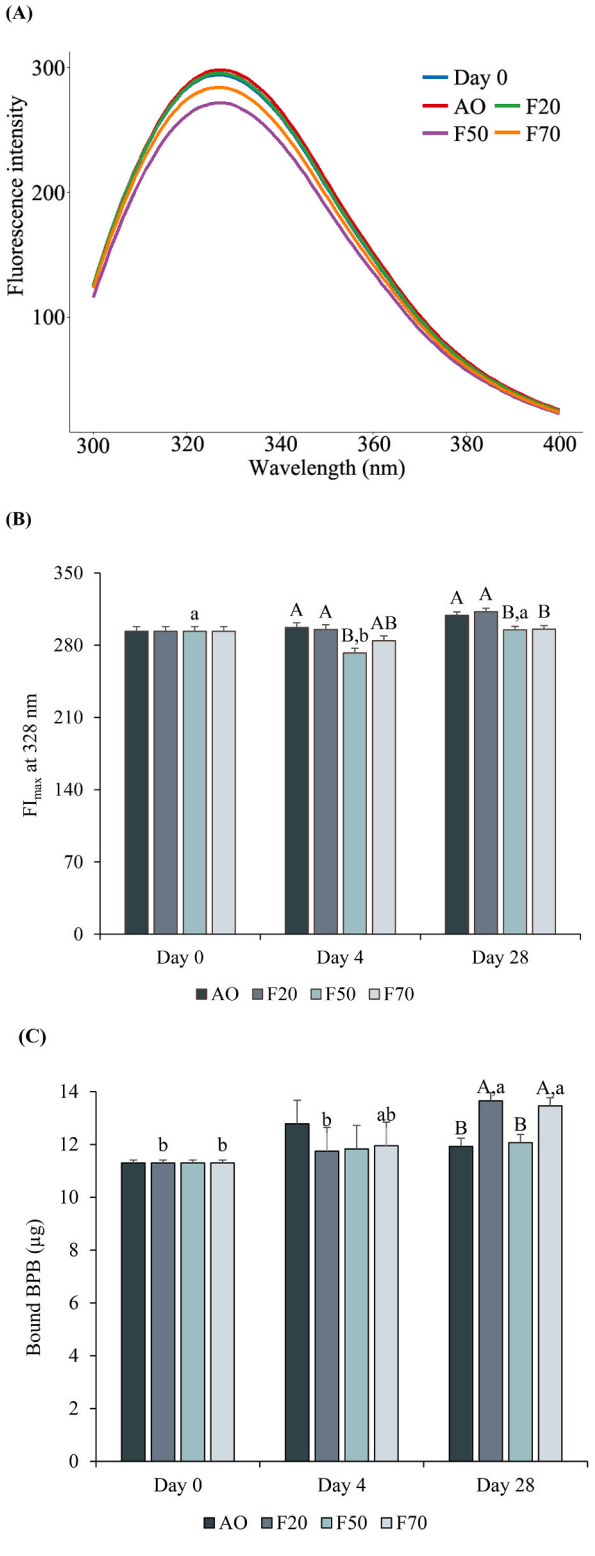

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Fig. 1. Endogenous fluorescence intensity of tryptophan on day 4 (A), maximum fluorescence intensity of tryptophan at 328 nm (B), and the content of bound bromophenol blue (BPB, μg, C) of myofibrillar proteins on days 0, 4, and 28 of aging.
AO, aged-only group; F20, beef frozen at −20 °C and then aged; F50, beef frozen at −50 °C and then aged; F70, beef frozen at −70 °C and then aged. Data are presented as mean ± standard error of the least square mean within a day (n = 12).
[A-B] Different uppercase letters indicate significant differences in mean between treatments in the same aging period ($P < 0.05$).
[a-b] Different lowercase letters indicate significant differences in mean between aging periods in the same treatment ($P < 0.05$).

groups than AO treatment on 14 and 28 days of aging due to the increased proteolysis because of enhanced cathepsin activities after freezing (Table 1, $P < 0.05$), as previously discussed. Although the F20, F50, and F70 treatments were subject to freezing-induced oxidative environments, such as the release of prooxidants after cellular disruption [25] and increased ionic strength [26], the antioxidative activities of biological peptides derived during aging might have prevented protein oxidation [24,27], resulting in no difference between AO and frozen-then-aged treatments.

All treatments showed a significant increase in carbonyl content on day 28 ($P < 0.05$). Although meat is rich in endogenous antioxidants, including carnosine, anserine, glutathione peroxidase, and superoxide dismutase [27], their activities gradually decrease during prolonged storage as the levels of prooxidants, such as metal ions and free radicals, increase [12]. This might have increased protein oxidation on day 28 in all four treatments.

3.3. Structural changes of proteins in beef

3.3.1. Tertiary structure of myofibrillar proteins in beef

Beef myofibrillar proteins had maximum fluorescence intensities (FI_{max}) at 328 nm when excited at 280 nm without any change in λ_{max}, indicating that there was no change in the microenvironment of tryptophan residues (Fig. 1A). The fluorescence spectra of beef on days 0 and 4 showed that aging beef for 4 days did significantly not affect FI_{max} (Fig. 1B, $P > 0.05$). On day 4, only F50 showed a significant decrease in FI_{max} at 328 nm ($P < 0.05$), whereas F20 and F70 showed no change compared to day 0 ($P > 0.05$). On day 28, F50 and F70 had a lower FI_{max} than AO and F20 ($P < 0.05$), although F50 had a higher FI_{max} on day 28 ($P < 0.05$), which was the same value as that on day 0.

The content of bound BPB, which indicates surface hydrophobicity, of beef myofibrillar proteins did not change significantly after freezing and aging on day 4 (Fig. 1C, $P > 0.05$). Unfolded hydrophobic residues on the protein surface can engage in hydrophobic interactions when they encounter distant residues [10]. Therefore, on day 4, the surface hydrophobicity of myofibrillar proteins might not have changed in F50 compared to day 0 due to occlusion of the unfolded residues during thawing for 2 days. However, on day 28, F20 and F70 showed higher surface hydrophobicity ($P < 0.05$) than AO and F50. Previous studies reported protein refolding during meat thawing due to the alleviation of denaturing factors [13,28]. Therefore, misfolding of myofibrillar protein upon refolding during aging in F20 and F70 could have resulted in the arrangement of the hydrophobic residues on the protein surface, leading to increased surface hydrophobicity.

Overall, only F50 showed significant unfolding of the originally buried tryptophan residues after freezing and thawing, although it exhibited a re-increase in fluorescence intensity on day 28. Although we expected cold denaturation to modify the hydrophobic interactions after freezing treatment, there was no alteration in the parameters used to monitor the tertiary structure, except for F50. This is further discussed in Section 3.3.2 with the results of the secondary structure of myosin and actin fractions.

3.3.2. Secondary structures of myosin and actin fractions in beef

Freezing-induced modifications in the secondary structures of the beef proteins were measured by CD spectroscopy (Fig. 2). We extracted myosin and actin fractions, two of the most prominent myofibrillar proteins (Supplementary Fig. 1), to track the denaturation behavior of proteins by freezing.

α-Helices, the most predominant component in meat proteins, are mainly found in myosin tails with a supercoiled helical structure [29], which is consistent with the higher α-helix content of the myosin fraction (Fig. 2A) than of the actin fraction (Fig. 2B). In both fractions, a decrease in α-helix content was observed on day 4 in all treatments ($P < 0.05$), and this reduction was more apparent in the frozen-then-aged groups than in the AO group. Hydrogen bonds between the carboxyl oxygens and amino hydrogens are the main forces that stabilize the α-helical structure [13]. As the temperature decreases, the number of hydrogen bonds between proteins and water molecules increases, leading to the exposure of hydrophobic residues and disruption of hydrogen bonds in the polypeptide backbone [5]. In the case of AO, proteolysis during four days of aging may have decreased the ordered and rigid structure by showing a further decrease in helical structure on day 28 in both myosin and actin fractions. F20 and F70 showed a re-increase in α-helix content in both the myosin and actin fractions. Although F50 also showed a re-increase in α-helix content on day 28 in the actin fraction, there was no change in the myosin fraction.

All treatments increased the β-turn in myosin on day 4 ($P < 0.05$), while only AO showed a further increase in the β-turn on day 28 ($P < 0.05$). However, in the actin fraction, there was no change in the β-turn content of the frozen-then-aged treatments ($P > 0.05$), whereas AO had a decreased β-turn content on day 4 ($P < 0.05$). F20 and F50 showed decreased β-turn contents on day 28 ($P < 0.05$), while AO showed no changes ($P > 0.05$). F70 showed no change in β-turn content in the actin fraction during all periods of aging ($P > 0.05$).

The β-sheet and random coil contents increased on day 4 in both fractions of all treatments ($P < 0.05$, Fig. 2). The loss of the helical structure could have increased the structural disorder and random coils, and resulted in typical open structures. Nevertheless, the

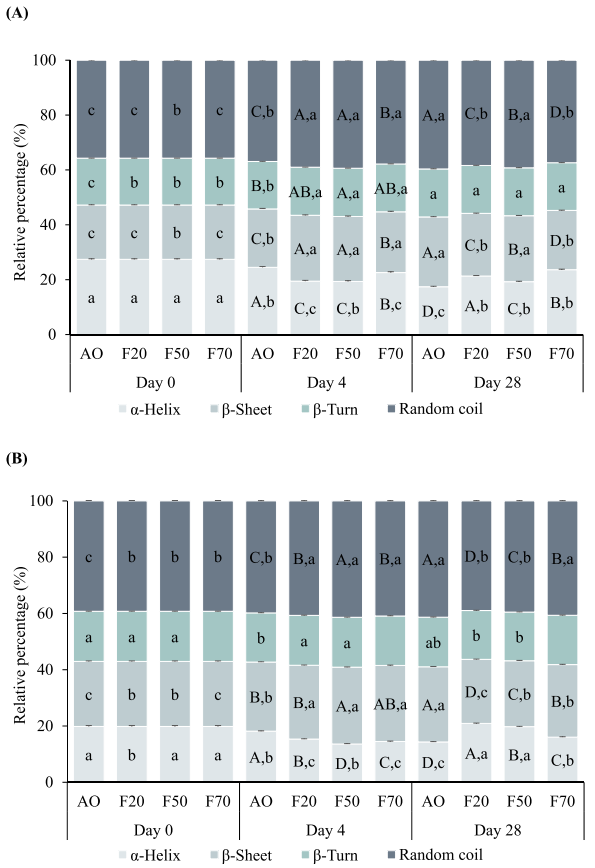

Fig. 2. Relative percentage (%) of the secondary structural components of the myosin (A) and actin (B) fractions on days 0, 4, and 28 of aging.

AO, aged-only group; F20, beef frozen at −20 °C and then aged; F50, beef frozen at −50 °C and then aged; F70, beef frozen at −70 °C and then aged. Data are presented as mean ± standard error of the least square mean within a day (n = 12).

[A-D] Different uppercase letters indicate significant differences in mean between treatments in the same aging period ($P < 0.05$).

[a-c] Different lowercase letters indicate significant differences in mean between aging periods in the same treatment ($P < 0.05$).

unfolded helical structures could have also been converted to β-sheet structures, which is another 'ordered' secondary structure that is stabilized by the intermolecular hydrogen bonds [30]. However, on day 28, only AO showed an increase in the β-sheet and random coil contents ($P < 0.05$), while F20 and F70 showed decrease ($P < 0.05$). F50 showed a decrease in the content of β-sheets and random coils only in the actin fraction ($P < 0.05$), while the myosin fraction did not change. These results are consistent with the changes in the α-helix content, indicating that the reduction in the helical structure by freezing was regenerated during aging; however, in the case of F50, the denaturation of the myosin helical tail was irreversible.

The changes in the secondary and tertiary structures of the myofibrillar proteins of F50 were different to those of F20 and F70 ($P < 0.05$). Only F50 showed a decrease in FI_{max} on day 4 ($P < 0.05$). Although F70 showed no significant difference in FI_{max} on day 4 compared to day 0 ($P > 0.05$), F50 and F70 had similar values, indicating that freezing-induced tryptophan unfolding also occurred during frozen storage at −70 °C. In the case of F20, no changes were observed in the tertiary structure ($P > 0.05$). However, all groups showed changes in the secondary structures of the myosin and actin fractions on day 4. This indicates that freezing-induced denaturation occurred in all the frozen-then-aged groups, even though the mechanism differed depending on temperature.

Freezing can induce protein denaturation *via* two mechanisms. The first is the formation of ice crystals, which changes significantly depending on freezing temperature. The freezing rate plays an important role in determining the distribution and size of ice crystals within frozen muscle fibers, which significantly affects protein denaturation. In general, previous studies have considered freezing meat at −20 °C as slow freezing [4,6]. A low freezing rate is generally considered to result in the formation of large and unevenly distributed extracellular ice crystals, causing subsequent damage to muscle fibers. Moreover, the large extracellular ice crystals increase the concentration of solutes in the surrounding non-frozen water fraction, resulting in osmotic water movement from the inside to the outside of the muscle fibers [26]. In contrast, fast freezing forms numerous small extracellular and intracellular ice crystals that are uniformly located between and within muscle fibers, resulting in less water migration of from the intracellular space [31]. In this study, the ice crystals generated in F20 might have been the largest and mainly located in the extracellular space. However, F70 might have had many small intracellular ice crystals, which caused the least ice crystal-induced damage to the muscles of the three

treatments. The size of ice crystals generated in F50 appears to be between those of F20 and F70.

The second one is cold denaturation. At low temperatures, the hydration of non-polar residues increases due to the decreased free energy penalty of entropically unfavorable interactions between water and hydrophobic groups [32]. This phenomenon is called 'cold denaturation' and causes changes in the interactions between proteins and water. Originally inaccessible hydrophobic amino acids, such as tryptophan, are solvated by water molecules at low temperatures, leading to the exposure of hydrophobic groups [9]. Therefore, the cold denaturation of proteins usually leads to changes in hydrophobic interactions and the disruption of hydrogen bonds. This can induce structural alterations in both tertiary and secondary protein structures. Although cold denaturation starts below 0 °C, freezing beef at −20 °C might not have been sufficient to induce significant unfolding compared to −50 °C, as Arsiccio et al. [5] reported that the protein structure was much more stable at 250 K (−23.15 °C) than at 230 K (−43.15 °C), where the native structure is partially maintained. Therefore, the formation of large extracellular ice crystals and increased solute concentration may have affected protein denaturation more than cold denaturation in F20. In contrast, as lower temperatures lead to more severe cold denaturation, cold denaturation might have been the main force altering protein structure in F70. However, since the size and distribution of ice crystals greatly affect the muscle fiber structure in meat systems [26], fewer changes in the tertiary structure might have been observed in F70 than in F50. In the case of F50, both the damage to the muscle structure caused by ice crystals and the effects of cold denaturation might have been somewhere between those of F20 and F70, resulting in changes in both tertiary and secondary structures.

Interestingly, the denatured secondary structure after freezing treatment was renatured in all treatments on day 28, except for the myosin fraction of F50, which showed irreversible denaturation of the secondary structure. The recovery of denatured myofibrillar proteins during thawing and chilled storage after freezing has also been previously reported [13,28]. It was reported that the cold denaturation-induced dissociation of protein molecules can be reversed upon incubation at certain temperatures [32]. Moreover, the melting of intracellular ice crystals could have also alleviated protein denaturation, since ice crystal-induced denaturation is one of the main causes of protein denaturation in meat [13]. These results can also explain the increase in surface hydrophobicity at day 28 in F20 and F70, which can be attributed to the refolding of myosin and actin. Moreover, F50 also showed an increase in FI_{max} at day 28

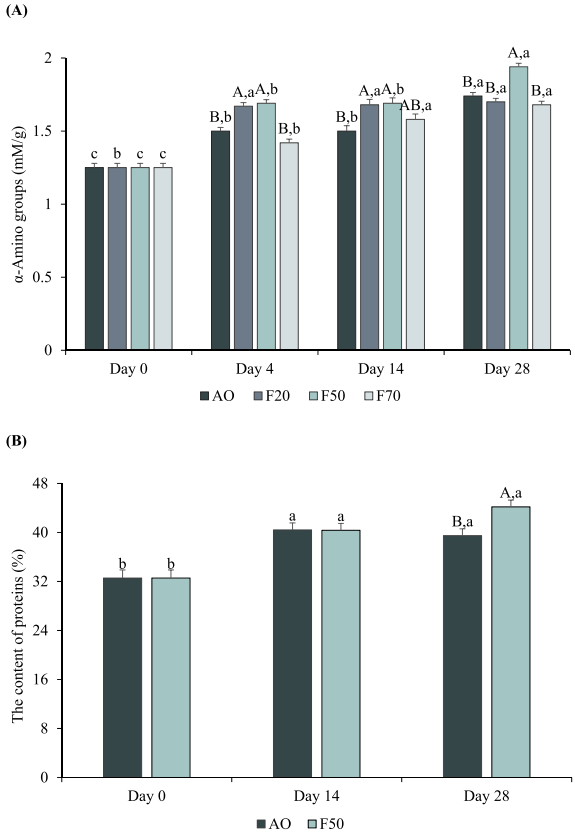

Fig. 3. α-Amino group content (mM/g, A) and the content of digested proteins under 3 kDa in the digesta samples (%, B) on days 0, 4, 14, and 28 of aging.

AO, aged-only group; F20, beef frozen at −20 °C and then aged; F50, beef frozen at −50 °C and then aged; F70, beef frozen at −70 °C and then aged. A, data are presented as mean ± standard error of the least square mean within a day (n = 12); B, data are presented as mean ± standard error of the least square mean within a day (n = 6).

[A-B] Different uppercase letters indicate significant differences in mean between treatments in the same aging period ($P < 0.05$).

[a-c] Different lowercase letters indicate significant differences in mean between aging periods in the same treatment ($P < 0.05$).

compared to day 4, which may also be due to the renaturation of actin and other myofibrillar components.

The reason the recovery was irreversible only in F50 seems to be that protein denaturation occurred to a greater extent because of both ice crystals and cold denaturation. Why, then, was the denaturation of the actin fraction reversible during F50 aging? We speculate that this difference between myosin and actin molecules might could be attributed to the number of charged residues on the surface of the proteins. Myosin, which predominantly exists in the A-band, typically has charged groups on the outside, whereas actin in the I-band has them on the inside [33,34]. Because of this, myosin molecules exhibit a higher capacity to bind water, and thus, we assume that myosin was more susceptible to cold denaturation, which changes the interaction between water and protein molecules and exposes hydrophobic groups that were originally located in the protein backbone. Since actin is less susceptible to cold denaturation than myosin, cold denaturation in the actin fraction might have been partially reversible.

3.4. *In vitro beef protein digestibility*

The unfolding of proteins by processing can increase the chances of degradation during digestion through the enhanced accessibility of digestive enzymes to the originally buried cleaving sites, resulting in the improvement of protein digestibility [10]. Therefore, we consider that the loosened structure can have higher proteolytic availability than the compact structure, contributing to the enhanced protein digestion.

We employed two parameters, the content of α-amino groups in the digesta (Fig. 3A) and the content of the digested proteins under 3 kDa (Fig. 3B), to evaluate *in vitro* protein digestibility. Since digested proteins under 3 kDa are believed to be absorbed in the small intestine [10], we used a centrifugal filter (3 kDa MWCO) to determine protein digestibility.

F20 and F50 had more α-amino groups than AO on days 4 and 14 (Fig. 3A, $P < 0.05$), while there was no difference between F70 and AO ($P > 0.05$). On day 28, F50 had the highest α-amino group content, and there were no significant differences between the other three treatments. The contents of α-amino groups did not change in F20 and F70 from days 14–28 ($P > 0.05$), while AO and F50 showed an increase in α-amino groups until day 28 ($P < 0.05$). Therefore, beef aged for longer than 14 days was only effective in AO and F50 to increase protein digestibility.

We compared the content of digested proteins under 3 kDa of AO and F50 on days 14 and 28 (Fig. 3B). Although there was no significant difference on day 14, F50 had a higher protein content (<3 kDa) on day 28 than AO ($P < 0.05$). Freezing beef at −50 °C and aging for 28 days can increase the protein content that can potentially be absorbed in the small intestine after digestion.

In the SDS-PAGE electrophoretogram of the digesta (Fig. 4 and Supplementary Fig. 2), all treatments on day 28 had fewer bands than on day 0. Actin, troponin-T, and myosin light chain B bands appeared clearly on day 0. However, after aging for 28 days, these bands had lower intensities than on day 0 in all treatments. The myosin light chain and actin bands almost disappeared in the frozen-then-aged treatments, particularly in F50, which had no actin band. Therefore, it seems that the appropriate condition to improve protein digestibility in this study was F50 on day 28.

Although all frozen-then-aged treatments showed 10% TCA-soluble α-amino groups during aging, there was no significant difference in the α-amino groups of the digesta between AO, F20, and F70 on day 28. It is obvious that the freezing treatment prior to aging increased proteolytic behavior, which can also influence digestive behavior. However, based on the results described above, the high protein digestibility of F50 throughout the aging period seems to be mainly affected by freezing-induced structural changes. The irreversible denaturation in the myosin fraction and increased random coil structure, which indicate structural disorder, could have improved substrate accessibility for digestive enzymes.

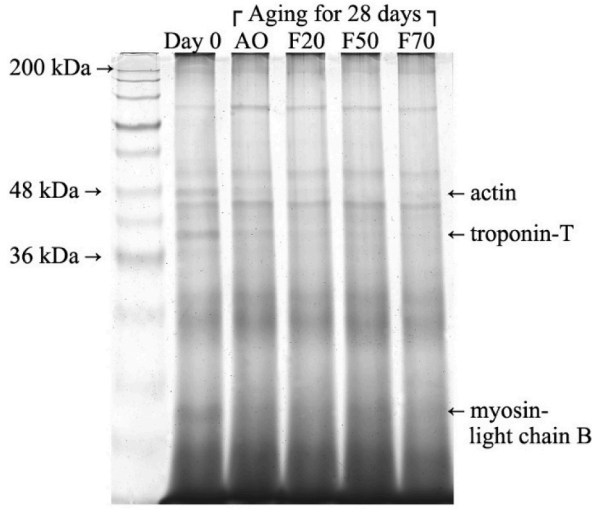

Fig. 4. SDS-PAGE electrophoretogram of beef samples (A) and digesta samples (B) aged for 28 days.
AO, aged-only group; F20, beef frozen at −20 °C and then aged; F50, beef frozen at −50 °C and then aged; F70, beef frozen at −70 °C and then aged.

In this study, the actin band remained after *in vitro* digestion of the samples on day 0. Actin has been reported to be refractory to digestive proteolysis due to its digestive-resistant core with 33–34 kDa of molecular mass [35]. This might be due to the actin-actin interactions in F-actin or the actin-myosin interactions so that the cleaving sites are buried. The low digestive accessibility of actin can hinder the overall digestion of myofibrillar proteins, particularly gastric digestion [10]. The postmortem formation of actomyosin cross-bridges between myosin and actin [29] also makes it difficult for digestive proteases to actin cleavage sites. Therefore, an increase in myosin degradation can enhance the digestive accessibility of actin. Myosin has regions that are particularly susceptible to digestion by trypsin and chymotrypsin in its polypeptide chains in the helical tail and globular head [8]. Since the helical structure existing in the globular head and tail of the myosin molecule remains reduced in F50 on day 28 (Fig. 2A), the digestive accessibility of myosin might have been improved, resulting in the disappearance of the actin band in the SDS-PAGE electrophoretogram of the digesta samples.

In addition, compared to day 4, immediately after freezing and thawing, the digestibility increased more on day 28 for F50, indicating that aging further improved the digestive accessibility. Since AO shows a gradual loss of α-helical structure and an increase in random coils, aging itself seems to contribute to structural disorder during protein degradation by the activities of endogenous proteases.

Overall, the three freezing temperatures had different freezing denaturation and refolding mechanisms during 28 days of aging, resulting in different proteolytic behaviors during *in vitro* digestion. The ice crystal-induced physiological disruption in the structure and the occurrence of cold denaturation in F50 appear to induce irreversible myosin denaturation, resulting in increased digestive susceptibility of myofibrillar proteins. Moreover, accelerated protein degradation during aging after freezing could have also contributed to the structural disorder and looseness of the proteins.

4. Conclusion

This study employed three prefreezing temperatures and aging periods to monitor changes in the protein structure and digestibility of beef. Prefreezing enhanced cathepsin B activity and protein degradation in frozen-then-aged treatments. Interestingly, prefreezing temperatures had different tendencies to induce secondary and tertiary structural changes in myofibrillar proteins. Only F50 showed a decrease in tryptophan fluorescence intensity on day 4. Although all frozen-then-aged treatments reduced the α-helical structure and increased the β-sheet and random coil contents on day 4, a re-increase in the α-helix content was observed in the myosin and actin fractions of F20 and F70 on day 28. However, in the case of F50, only the actin fraction showed protein refolding, and the myosin fraction had irreversible denaturation of the secondary structure on day 28. This irreversible unfolding of myosin molecules in F50 resulted in a higher content of digested proteins under 3 kDa than AO, with the disappearance of actin bands in the SDS-PAGE electrophoretogram of the digesta. These findings show that different mechanisms can be used to explain the structural changes in proteins depending on the freezing temperature.

Therefore, the prefreezing treatment at −50 °C and subsequent aging of beef can increase protein digestibility in infants and improve the nutritional quality of beef. The beef source with improved protein digestibility can offer less risk of digestive problems such as diarrhea and allergic reactions for infants who initially experience semi-solid complementary foods. Thus, the evaluation of the effect of freezing-then-aging treatment on the protein digestibility of beef using a dynamic digestion model in the future study can improve the understanding of the digestive behavior of beef proteins in various digestive conditions of infants (premature or full-term, by age, etc.).

Author contribution statement

Seonmin Lee: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kyung Jo, Hyun Gyung Jeong, Yun-Sang Choi: Performed the experiments.

Samooel Jung: Conceived and designed the experiments; Analyzed in interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15611.

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