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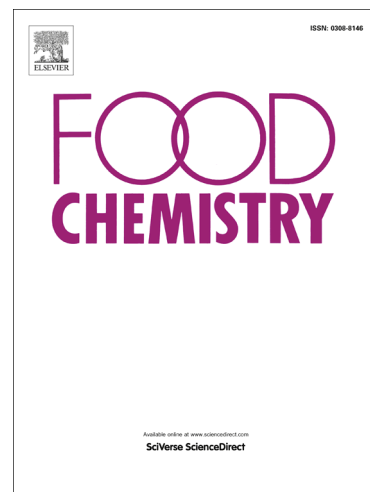
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On the origin of thaw loss: Relationship between freezing rate and protein denaturation

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Abstract

The role of protein denaturation in formation of thaw loss is currently not well understood. This study investigated denaturation of myofibrillar and sarcoplasmic proteins of pork loins caused by freezing-thawing in relation to freezing rate. Compared to fast freezing, slow freezing caused 28% larger thaw loss, decreased water-holding capacity of myofibrils and increased surface hydrophobicity, indicating more pronounced denaturation of myofibrillar proteins. We here propose a model: In slow freezing protons are concentrated in the unfrozen water resulting in reduced pH in proximity of structural proteins causing protein denaturation. In parallel, large ice crystals are formed outside of muscle fibers resulting in transversal shrinkage. In fast freezing small ice crystals trap protons and cause less severe protein denaturation and reduced thaw loss. Differential scanning calorimetry and tryptophan fluorescence spectra indicated sarcoplasmic protein denaturation in drip due to freezing-thawing. However, sarcoplasmic protein denaturation was independent of freezing rate.

Keywords: Meat, Ice crystal formation, Purge loss, Myofibril structure, DSC

1. Introduction

Freezing has been widely applied in the meat industry, since it can prevent microbial deterioration and preserve meat and meat products for long time storage (Coombs, Holman, Friend, & Hopkins, 2017). However, the freezing process inevitably leads to some quality loss, particularly water loss during

thawing which might result in both economic and nutritional disadvantages considering the losses of weight and some valuable nutrients.

Water accounts for around 75% in muscle tissue, and the majority of water in the muscle fiber is trapped within the muscle structure, especially within and between the myofibrils (Huff-Lonergan & Lonergan, 2005). Water-holding capacity of meat in terms of thaw loss, drip loss and cooking loss has been reported to decrease following freezing and thawing (Aroeira, et al., 2016; Kim, Kim, Seo, Setyabrata, & Kim, 2018), presumably attributed to the mechanical damage caused by ice crystallization and protein denaturation (Calvelo, 1981).

It is generally believed that ice crystal formation is responsible for thaw loss and that the size and distribution of ice crystals inside and outside the muscle fibers are mainly affected by the freezing rate (Hamm, 1986; Li, Zhu, & Sun, 2018). Slow freezing has often been reported to produce more thaw loss compared to fast freezing (Calvelo, 1981; Hamm, 1961; Petrović, Grujić, & Petrović, 1993). Freezing of muscle usually begins in the extracellular area after the temperature has declined below $-1\text{ }^{\circ}\text{C}$. The formation of extracellular ice is immediately followed by an increase in the concentration of solutes in the surrounding non-frozen water, creating a pressure for water to be moved osmotically from inside to outside of the muscle fibers. Consequently, slow freezing will form large ice crystals distributed unevenly in the extracellular space (Bevilacqua, Zaritzky, & Calvelo, 1979; Bevilacqua, & Zaritzky, 1980; Grujić, Petrović, Pikula, & Amidžić, 1993). However, fast freezing results in numerous small intra- and extracellular ice crystals which are located uniformly within and between the muscle fibers and causes less migration of intracellular water to outside of fibers (Bevilacqua, et al., 1979; Kim, et al., 2018). Water within or between myofibrils have to migrate across the sarcolemma membrane to reach the extracellular spaces between the muscle fibers before reaching the surface of meat where it can be lost

as drip or exudate (Hughes, Oiseth, Purslow, & Warner, 2014). Water in extracellular ice crystals in slowly frozen meat has already crossed the sarcolemma. Upon thawing, the water originating from extracellular ice crystals is already in the extracellular space and thereby reach the meat surface more easily. Additionally, mechanical damage from shrinkage and distortion of muscle fibers due to the formation of large ice crystals in slow freezing has been suggested to have a large influence on the reabsorption of water by muscle fibers during thawing, compared to small ice crystals in fast freezing meat (Hamm, 1986).

The idea that freezing and thawing induces protein denaturation was proposed more than half a century ago, and initial studies concluded that the process itself does not cause excessive denaturation of meat proteins (Hamm, 1961). Any protein denaturation that takes place during freezing potentially contributes to a decrease in water-holding capacity of meat resulting in the formation of thaw loss. Protein denaturation due to freezing has traditionally been attributed to an increased concentration of solutes in the unfrozen water phase of meat tissue (Calvelo, 1981; Sikorski, Olley, Kostuch, & Olcott, 1976). Nevertheless, several studies have over the years refuted the idea that protein denaturation during freezing-thawing is significantly contributing to the decrease in water-holding as reviewed by Hamm (1986) and Leygonie, Britz & Hoffman (2012). New analytical tools to assess the degree of freeze damage in meat are constantly being developed (Egelandsdal, et al., 2019), thus expanding our understanding of the mechanisms involved in generation of thaw loss. Denaturation of myofibrillar proteins has been reported in frozen-thawed meat by observing an increased surface hydrophobicity, reduced denaturation enthalpy and decreased protein solubility (Qi, Li, Chen, Gao, Xu, & Zhou, 2012; Xia, Kong, Xiong, & Ren, 2010). Wagner & Anon (1985) reported an effect of freezing rate on myofibrillar proteins denaturation in beef by observing a greater loss of Mg^{2+} and Ca^{2+} -ATPase activity and a lower denaturation enthalpy for slow freezing compared to fast freezing rate. However, other

studies on freezing rate did not observe a significant influence on protein denaturation in terms of protein solubility and surface hydrophobicity (Farouk, Wieliczko, & Merts, 2003; Yu, et al., 2010) or on denaturation enthalpy (Ngapo, Babare, Reynolds, & Mawson, 1999), and these authors attributed the different amounts of thaw loss to the differences of the mechanical damage in muscle fibers due to different sizes and locations of ice crystals between fast and slow freezing. The importance of protein denaturation for the formation of thaw loss is thus still not well understood, and there is a lack of agreement in literature on the relative importance.

In the current study, we hypothesize that the freezing rate corresponding to slow and fast freezing is strongly related to protein denaturation, which in turn plays a major role for formation of thaw loss. We investigated the denaturation of myofibrillar and sarcoplasmic proteins in porcine *longissimus thoracis et lumborum* (LTL) muscle. Myofibrillar proteins denaturation was assessed by measuring water-holding capacity of myofibrils, surface hydrophobicity of myofibrils and differential scanning calorimetry (DSC) analysis of meat samples. Sarcoplasmic proteins denaturation was indicated by intrinsic fluorescence scanning and DSC analysis of meat drip.

2. Materials and methods

2.1. Sample processing

Pigs were slaughtered at HK-Ruokatalo, Forssa Slaughterhouse in Finland, and then six pork loins from different animals were excised at 6 h *post-mortem* and transported to the meat laboratory at University of Helsinki at 10 h *post-mortem*. The LTL muscle was isolated and cut into 12 steaks (around $9 \times 6 \times 2$ cm along the muscle length), individually weighed (average weight of 88.8 ± 11.1 g per steak) and vacuum packaged following trimming of visible connective tissue and external fat and stored at 2 ± 1 °C until 24 h *post-mortem*. These steaks from each LTL muscle (n=72 for all steaks) were numbered 1 to 12

and then assigned to one of three treatments: 1) non-frozen control, 2) fast and 3) slow freezing. The non-frozen control (steak 1, 4, 7 and 10, n=24) were stored at 2 ± 1 °C in a cold room (Huurre, Vantaa, Finland); the steaks (2, 5, 8 and 11, n=24) and the steaks (6, 9 and 12, n=24) were assigned to fast and slow freezing, respectively. Fast freezing was conducted in a -80 °C freezer (Ultra Low, SANYO, Japan) and samples were frozen on a metal plate, whereas slow freezing was performed in a -18 °C walk-in freezer (Huurre) and samples were frozen on polystyrene foam plates placed on shelves. The freezing was mainly longitudinally to the direction of muscle fibers. The internal temperature for both fast and slow freezing groups during freezing were monitored by type K thermocouples connected to a data logger (Honeywell DPR-3000) at intervals of 1 min (Fig. 1). After 24 h of storage at -18 °C, slow freezing samples were transferred to the -80 °C freezer (in which the fast freezing samples were frozen) and analysis was performed within two weeks. The slow frozen samples were stored at -80 °C to minimize any effect of frozen storage time considering that there might be recrystallization of ice at -20 °C. This procedure ensures that the study focus on the rate of freezing rather than any effects of frozen storage temperature.

Following 24 h of storage, non-frozen control (steak 1 and 4) were sampled for purge loss at 48 h *post-mortem*, water-holding capacity of myofibrils, and surface hydrophobicity of myofibrils and DSC analysis of meat. The fast and slow freezing groups (steak 2, 3, 5 and 6) were thawed at 2 ± 1 °C for 24 h, and then conducted the same determinations with the non-frozen control. For the purge loss at 72 h *post-mortem*, intrinsic fluorescence scanning and DSC analysis of meat drip, non-frozen control (steak 7 and 10) stored at 2 ± 1 °C after 48 h of storage and frozen samples (steak 8 and 11 for fast freezing; steak 9 and 12 for slow freezing) thawed at the same temperature for 48 h were taken for sampling.

The ultimate pH at 24 h *post-mortem* was 5.5 ± 0.1 measured by an insertion electrode (Mettler-Toledo Inlab 427). The average values of L^* , a^* and b^* were 52.9 ± 1.8 , 7.5 ± 1.5 and 4.5 ± 1.6 , respectively, measured by a Minolta Chroma meter CR-400 (Minolta Camera Co. Ltd., Osaka, Japan) set at D65 illuminant with a 8 mm diameter of aperture. A white tile (C: $Y = 93.6$, $x = 0.3130$, $y = 0.3193$) was used to calibrate for the instrument.

2.2. Purge loss

Meat steaks were weighed at 24 h *post-mortem* and recorded as the initial weight before freezing. Following 24 h and 48 h of storage at 2 ± 1 °C, the bags from non-frozen group were opened and the weight of meat steaks was recorded after removing the superficial liquid with filter paper. Frozen samples were thawed at 2 ± 1 °C for 24 h and 48 h followed by weighing the meat steaks individually. Purge loss was determined by calculating the difference between the initial weight and the weight after storage for 24 h and 48 h (shown as percentage) and thus included the thaw loss of frozen-thawed samples.

2.3. Isolation of myofibrils

Meat samples (2 g) in 20 mL of cold washing buffer (75 mM KCl, 100 mM MES, 2 mM $MgCl_2$, 2 mM EGTA, pH 5.5) were homogenized using an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany, 10 s at 13,500 rpm), and were then centrifuged at $10,000 \times g$ for 10 min at 4 °C. Myofibrils pellets (around 1.8 g) was obtained after being washed two times with washing buffer to remove soluble proteins. The composition of myofibrils did not differ between fresh, fast and slow frozen samples (Supplemental material 1).

2.4. Water-holding capacity of myofibrils

Water-holding capacity of myofibrils was measured according to previous method described by Zhang & Ertbjerg (2018). The relative water-holding capacity of myofibrils was expressed as the amount of water held by 1 g of myofibrillar protein.

2.5. Surface hydrophobicity

Surface hydrophobicity of the myofibrils pellets was determined according to the method described by Liu, Arner, Puolanne, & Ertbjerg (2016) with minor modifications. The pellet was re-suspended in washing buffer and the protein content was measured by using the method of DC Protein Assay Kit. One mL of protein suspension (adjusted to 2 mg/mL) was mixed with 80 μ L of 1 mg/mL bromophenol blue (BPB) and subsequently incubated for 10 min at room temperature followed by centrifugation at 10,000 g for 3 min. For the control, 80 μ L of 1 mg/mL BPB was added to 1 mL of washing buffer. The supernatant obtained was diluted 10 times with washing buffer and the absorbance was measured at 595 nm. The amount of bound BPB per mg protein was calculated by:

$$\text{BPB bound } (\mu\text{g}) / \text{mg protein} = 80 \mu\text{g BPB} \times (\text{A control} - \text{A sample}) / \text{A control} / 2 \text{ mg protein.}$$

With A = absorbance at 595 nm

2.6. Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed both in the meat sample and purge drip as described previously by Voutila, Perero, Ruusunen, Jouppila, & Puolanne (2009) with some modifications. Measurements were operated in duplicate by using a DSC 823^e (Mettler Toledo AG, Greifensee, Switzerland). Around 30 mg of meat samples (or 30 μ L of purge drip) were weighed in the 40 μ L aluminum sample pan, and then the pans were hermetically sealed. The protein content in the drip was determined by reading absorbance at 280nm. The heating temperature from 25-95 $^{\circ}$ C at a heating rate of 5 $^{\circ}$ C \times min⁻¹ was programmed and then the heat flow was recorded using the software STARE

Evaluation Version 16.00 (Mettler-Toledo, Zurich, CH) connected to the DSC instrument. An empty sample pan was used as a reference. After DSC analysis, sample pans were pierced following by drying at 105 °C overnight to obtain the weight of the dry matter. The peak temperature (T_{peak}) and denaturation enthalpy (ΔH) were chosen to describe protein denaturation. The ΔH values were expressed as J g^{-1} (joules per gram dry matter).

2.7. *Intrinsic fluorescence measurement*

The intrinsic fluorescence emission spectra of meat drip (at 72 h *post-mortem*) were measured by a LS 55 Luminescence Spectrometer (PerkinElmer Inc., Waltham, MA, USA) as described previously by Estévez, Kylli, Puolanne, Kivikari, & Heinonen (2008) with minor modifications. Meat drip (from both fresh and frozen-thawed samples) was diluted in washing buffer, and was then adjusted to a final concentration of 0.015 mg/mL. The excitation was set at a wavelength of 295 nm, and the emission spectra were recorded from 300 to 400 nm with slit width of 7 nm and scanning speed of 240 nm/min.

2.8. *Exposure of myofibrils to combinations of different pH and ionic strength*

Myofibril pellets were isolated from five fast frozen LTL muscles by thawing steaks at 2 °C overnight and then homogenizing 20 g in 80 mL cold washing buffer following the method of 2.3. An aliquot of each muscle (2.2 g, resulting in five replicates) were exposed to different pH and ionic strength conditions by adding 30 mL of the following incubating buffers: (1) 0.15 M KCl, pH 5.5; (2) 0.42 M KCl, pH 5.4; (3) 0.69 M KCl, pH 5.3; (4) 0.96 M KCl, pH 5.1; and (5) 1.13 M KCl, pH 5.0; (6) 1.5 M KCl, pH 4.8. The buffers all contained 50 mM MES, 2 mM MgCl_2 , and 2 mM EGTA. The pellets were then centrifuged at $2400 \times g$ for 10 min followed by one more wash in the same buffer. The resultant pellets were resuspended in 20 mL of the same incubation buffer and vortexed at 4 °C for 10 mins (final exposure

to the pH and ionic strength condition). The pH value of the myofibrils suspension was measured at 4 °C. The exposure was followed by centrifuging at $2400 \times g$ for 10 min. To restore the pH 5.5 and low ionic strength, the myofibril pellets were subsequently washed with 25 mL cold washing buffer (pH 5.5) and centrifuged at $2400 \times g$ for 10 min. The washing was repeated once. The resultant myofibril pellets were used for determination of water-holding capacity, surface hydrophobicity and particle size. The particle size distribution of the pellets was determined by a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK). The refractive index was set to 1.46 and the absorption coefficient to 0.01. $D(v,0.1)$, and $D(v,0.9)$ represents that the size of the particle for which 10% and 90% of the sample is below the corresponding size, respectively. $D(3,2)$ is the mean diameter in surface, and $D(4,3)$ is the mean diameter in volume.

2.9. Statistical analysis

To study the effect of freezing and freezing rate on myofibrillar and sarcoplasmic proteins of pork LTL muscle, two steaks from each muscle were represented at every treatment. For each of the six muscles, duplicates were done for purge loss, four replicates were done for water-holding capacity, surface hydrophobicity and DSC measurement, and six replicates were done for intrinsic fluorescence. Five independent repetitions of the exposure of myofibrils to combinations of different pH and ionic strength were performed. One LTL muscle was used for each repetition resulting in five determinations of water-holding capacity, surface hydrophobicity and particle size measurement. Data analysis was carried out by using general linear model in the IBM SPSS Statistics 24 software. Treatment (non-frozen, fast and slow freezing) was arranged as fixed factor, and animal number was arranged as a random factor. Bonferroni test was conducted to evaluate the significant differences between group means at a level at $P < 0.05$.

3. Results

3.1. Center temperature decline

The characteristic freezing time (t_c), defined as the time the meat temperature changes from $-1\text{ }^\circ\text{C}$ (beginning of freezing) to $-7\text{ }^\circ\text{C}$ (80% of water being frozen), is a measure of how the freezing rate affect their sizes and locations. Freezing at a $t_c < 15\text{ min}$ forms intra- and extracellular ice with small diameter, whereas for longer characteristic time ($t_c > 23\text{ min}$), large ice crystals are formed in the extracellular area during freezing of beef (Bevilacqua, et al., 1979). In the present study, the observed t_c in the center of pork LTL steaks were 12 min for the fast frozen and 174 min for the slow frozen muscle (Fig. 1), and the used freezing rates, therefore, belong to categories of fast and slow freezing known to result in different sizes and distributions of ice crystals (Grujić, et al., 1993; Martino, Otero, Sanz, & Zaritzky, 1998).

3.2. Purge loss

Purge loss of fresh pork LTL muscle was sampled after 24 and 48 h of chilled storage, and frozen-thawed muscle after thawing-storage for 24 and 48 h. As expected the 48 h purge loss values were larger. Taken together, the water loss of the three treatment groups was in the following order: fresh < fast freezing < slow freezing. Freezing-thawing increased ($P < 0.01$) purge loss of 24 h samples from 4.5% (fresh) to 5.8% (fast freezing) and 7.3% (slow freezing). The largest ($P < 0.01$) purge loss was clearly observed in slow freezing group (Fig. 2 A), being on average 28% greater in slow compared to fast freezing.

3.3. Water-holding capacity and surface hydrophobicity of myofibrils

Freezing and freezing rate both significantly affected the water-holding capacity of myofibrils (Fig. 2B), and fresh group showed the largest value of 8.0 g H₂O/g protein in comparison with the values of 7.1 and 6.8 g H₂O/g protein in the fast and slow freezing group, respectively.

The surface hydrophobicity of myofibrils was smallest ($P < 0.01$) in the fresh group in comparison with the two frozen-thawed groups (Fig. 2C). A significant effect of freezing rate was observed in which myofibrils extracted from slow freezing samples showed a larger value ($P < 0.05$) of surface hydrophobicity compared to that of fast freezing samples.

3.4. DSC

Representative DSC curves are shown in Supplemental material 2 and the peak temperature (T_{peak}) and denaturation enthalpy (ΔH) in meat and purge drip from the fresh and two frozen-thawed groups are shown in Table 1. Three endothermic peaks were observed in DSC curves for meat, and one endothermic peak for purge drip. In meat the process of freezing decreased the peak denaturation temperature; the fresh group showed slightly higher ($P < 0.01$) temperatures of all three peaks compared to the two frozen-thawed groups, whereas there were no significant differences between fast and slow freezing samples. Similarly, the fresh group had a higher ($P < 0.05$) denaturation enthalpy of 1.54 J g^{-1} for the first peak in comparison with 1.47 and 1.42 J g^{-1} for the fast and slow freezing group, respectively. However, the denaturation enthalpy for the third peak was observed to be lower ($P < 0.01$) in fresh meat as compared to frozen-thawed meat. The denaturation enthalpy for the second peak was lower ($P < 0.01$) in the slow freezing group, whereas no significant differences were found between fresh and fast freezing groups. A main effect of freezing on the denaturation enthalpy of purge drip was observed (Table 1). Compared to purge drip from fresh samples, frozen-thawed groups showed lower ($P < 0.01$) denaturation enthalpy, whereas no effect of freezing rate was observed. The protein content in drip collected from fresh meat was 138 mg /mL , and did not differ ($P > 0.05$) in comparison with the protein contents of 138 and 135 mg /mL in the drip from fast and slow freezing group, respectively.

3.5. Fluorescence emission spectra

The intrinsic emission fluorescence spectra of purge drip collected from fresh, fast and slow freezing pork steaks is shown in Fig. 3. A clear difference ($P < 0.01$) of fluorescence spectra was observed between drip from fresh and frozen-thawed meat, whereas fast freezing showed a similar fluorescence spectra as slow freezing. The maximum fluorescence emission wavelength did not shift significantly after the treatment of freezing and thawing. However, the fluorescence intensity (FI_{\max}) was significantly increased in the two frozen-thawed groups as compared to the fresh group.

3.6. Exposure of myofibrils to combinations of different pH and KCl concentrations

In this experiment we simulated the exposure of myofibril proteins to combinations of lower pH and higher ionic strength as hypothesized to occur in a freezing-thawing cycle. Based on the assumption that $> 90\%$ of the water is frozen below $-20\text{ }^{\circ}\text{C}$, this could theoretically result in a > 10 -fold increase in ionic strength and proton concentration in frozen meat. The relative high buffer capacity of myofibrillar proteins would, however, partly absorb the excess protons thereby lowering the pH decline. To obtain further information on the combined effect of pH and ionic strength, we exposed myofibrils to combinations of pH (from 5.5 to 5.0) and ionic strength (KCl concentration from 0.15 to 1.5 M), to investigate the effects on water-holding capacity, surface hydrophobicity and particle size of myofibrils. The combinations of lower pH and higher KCl concentration significantly decreased water-holding capacity, increased surface hydrophobicity and increased particle size onwards from around pH 5.2 and 1 M KCl (Table 2).

4. Discussion

4.1. A model for the effect of freezing rate and ice crystal formation on amount of thaw loss

Freezing of meat has been reported to produce a large water loss upon thawing. The rate of ice crystal formation is depending on the freezing rate and has a significant influence on the amount of thaw loss. In general, slow freezing results in more thaw loss than fast freezing (Hamm, 1961, Ngapo et al., 1999; Kim, et al., 2018). In the present study, compared to a fast freezing rate, slow freezing resulted in 28% more thaw loss, as well as decreased water-holding capacity of myofibrils and increased surface hydrophobicity of myofibrils, indicating that slow freezing induced pronounced denaturation of myofibrillar proteins. We here propose a model of the influence of protein denaturation on the formation of thaw loss and the relationship with the rate of ice crystals formation due to freezing rate (Fig. 4). Freezing will start extracellularly and consequently water migrates from inside of muscle fibers to outside. The water migration will result in dehydration and transversal shrinkage of muscle fibers and myofibrils. Solutes become more concentrated in the unfrozen water phase outside the ice crystals, also resulting in an increased concentration of protons, and thereby a decreased of pH of the remaining liquid water. The water-accessible surface area of structural proteins, such as the actin and myosin filaments within the myofibril, will thus be exposed to lower pH and higher ionic strength during growth of ice crystals, resulting in protein denaturation. Upon thawing, the dehydrated and denatured structural proteins will reabsorb less water and hence influence the amount of thaw loss. Freezing at fast rate forms within a short time small ice crystals distributed inside and outside of fibers in parallel with moderate transversal shrinkage of muscle fibers. We speculate that some protons are trapped inside fast growing small ice crystals, which may result in less decline of pH in the unfrozen water in fast freezing. However, at a slow freezing rate, larger parts of the water migrates from inside to outside of fibers to form large extracellular ice crystals, leading to a more severe transverse shrinkage of the fibers. Slow freezing, therefore, cause a higher concentration of solutes and protons in the unfrozen water and a corresponding lower pH as compared to fast freezing rate. Furthermore, the exposure time of myofilaments to the high concentration of solutes would be expected to be greater in slow freezing than fast freezing. Lower pH in combination

with greater ionic strength in the unfrozen water in slow freezing is, therefore, hypothesized (Fig. 4) to lead to more severe protein denaturation including unfolding as evidenced by increased surface hydrophobicity of myofibrils (Fig. 2C) which in turn reduce the amount of water that can be held by myofibrillar proteins (Fig. 2B), and thus slow freezing leads to a larger water loss upon thawing than fast freezing (Fig. 2A and 4).

We would like to stress that although the proposed model points to myofibrillar protein denaturation as the driving force of thaw loss formation, the model does not exclude that some of the drip is produced due to the direct effect that some of the water in ice crystals outside the fibers upon thawing migrates to the meat surface. The contribution of increasing ionic strength in the non-frozen water to protein unfolding may also be significant. It is thus well known that at ionic strength above 0.25 M NaCl, myosin filaments are progressively dissociated to myosin molecules (Offer & Knight, 1988a) and an effect on water-holding of selective binding of chloride ions to the shaft of myosin filaments was proposed. As the ionic strength exceeds 0.5 M in the liquid water during freezing-thawing (Offer & Knight, 1988b), it may contribute to development of thaw drip. However, salting before freezing appears to reduce the intracellular damages caused by freezing (Jiang, Jia, Nakazawa, Hu, Osako, & Okazaki, 2019), hence it can be questioned if the mechanism of increased thaw loss is a direct effect of increased ionic strength in the non-frozen water.

A speculation in the model (Fig. 4) is on a pH induced denaturation (in combination with ionic strength) of structural proteins in the freezing-thawing process. In freezing there is a substantial transversal shrinkage of the fibers (Koonz & Ramsbottom, 1939) to less than half of the original cross-sectional area (Bevilacqua et al., 1979) due to growth of extracellular ice crystals. As a result it is to be expected that protons will concentrate in the remaining intracellular liquid water. On the assumption that >90% of the

water is frozen this could theoretically result in a >10-fold increase in proton concentration, however, the buffer capacity of meat proteins would absorb a large part of the excess protons. Temporarily, during freezing and thawing, the water-accessible surface areas of the structural proteins within the myofibrils would then be exposed to a lower pH value. Exposure to lower pH, e.g. in pale, soft and exudative (PSE) meat, the denaturation of myofibrillar proteins caused by low pH combined with high temperature is linked to high drip loss (Liu, et al., 2016). The combination of a lower pH (around 5.2) and a higher KCl concentration (around 1 M) (Table 2), showed protein denaturation of a comparable extend to that observed in slow freezing (Fig. 2) evaluated as water-holding capacity and surface hydrophobicity. The protein changes in myofibrils in slow freezing could thus be reproduced by exposure of myofibrils to combinations of lower pH and higher ionic strength. Fast freezing will result in less transversal shrinkage, and additionally we suggest that some protons are trapped inside the ice crystals. Together with mechanical compression of the protein structure this offer an explanation of why thaw loss is less in fast freezing. In water protons exist as the hydronium (H_3O^+) ion. It has been shown that the hydronium ion can migrate in ice and both theoretical and experimental studies have predicted that excess protons at low temperature can be trapped in bulk ice (Park , Lin, & Paesani, 2014), although this was never shown in frozen meat.

Zhang et al., (2018) observed decreased water-holding of myofibrils from frozen-thawed pork. Also Petrović, et al. (1993) found decreased water-holding capacity of meat and solubility of myofibrillar proteins at slow freezing rate. These observations give direct evidence of pronounced denaturation of myofibrillar proteins due to freezing, and strongly suggest the involvement of myofibrillar proteins denaturation in the formation of thaw loss. In parallel with the loss of water-holding capacity in this study, the surface hydrophobicity of myofibrils observed in the slow freezing group was 24% larger than that of fresh samples, in comparison with 16% larger in the fast freezing group (Fig. 2C). Surface

hydrophobicity has been used to estimate protein denaturation, and factors such as pH could lead to changes in the hydrophobic interactions of protein structure thus influencing hydrophobicity. In agreement, previous studies on freezing found increased values of surface hydrophobicity, suggesting an unfolding of myofibrillar proteins (Chan, Omana, & Betti, 2011). Thus, both water-holding capacity and surface hydrophobicity data in our study indicate roles of freezing and freezing rate on considerable denaturation of myofibrillar proteins, in which slow freezing caused a larger damage to myofibrillar proteins than fast freezing. The nature of denaturation probably involves unfolding and aggregation of myofibrillar proteins as suggested by the increased surface hydrophobicity and particle size due to lower pH value and higher ionic strength (Table 2).

DSC has been used to study the thermal transitions of meat proteins in terms of freezing and thawing (Jia, He, Nirasawa, Tatsumi, Liu, & Liu, 2017; Jia, Nirasawa, Ji, Luo, & Liu, 2018). In general, three major endothermal peaks can be observed in meat tissue, and the transition indicating protein denaturation is often corresponding to the peak temperature and denaturation enthalpy. The first peak has been ascribed to myosin, the middle peak in DSC thermograms to sarcoplasmic proteins and collagen denaturation, and the third peak was assigned to actin (Stabursvik & Martens, 1980). Huang, Liu, Xia, Kong, & Xiong (2015) observed shifts of peak temperatures and total enthalpy to lower values in pork dumpling filling with increased frozen storage time, and they attributed the lower values to decreased thermal stability and increased protein aggregation caused by ice crystallization. In the present study, lower values for peak temperature and enthalpy for myosin were observed in the frozen-thawed groups compared to fresh samples, whereas no effect of freezing rate was observed, suggesting that a considerable denaturation of myosin is taking place due to freezing and thawing. This suggests that the changes of myosin structure caused by different freezing rates are having a greater effect on the surface properties of myofilaments than on the thermal stability.

4.2. Effect of freezing on sarcoplasmic proteins

This is the first study reporting an effect of freezing on sarcoplasmic proteins measured in thaw drip by DSC. Only one major endothermal peak representing sarcoplasmic proteins was observed in the DSC curve (Table 1). The decreases of enthalpy values in two frozen-thawed groups suggest that freezing and thawing are causing considerable denaturation of sarcoplasmic proteins caused by freezing and thawing. However, the freezing rate in this study had no significant influence on sarcoplasmic protein traits, suggesting that sarcoplasmic proteins are more stable than myofibrillar proteins during the freezing-thawing process. The intrinsic fluorescence spectra has been used to estimate the changes of tryptophan residues considering its higher quantum yields and environmental sensitivity than tyrosine and phenylalanine (Royer, 2006). Conformational changes of protein structure is usually corresponding to the maximum wavelength (λ_{\max}) and fluorescence intensity (FI_{\max}) in the fluorescence spectra. Zhang, Li, Diao, Kong, & Xia (2017) reported that a slight increase of λ_{\max} from 330.4 nm to 331 nm in myofibrillar proteins due to freezing and thawing suggests an exposure of the buried tryptophan to a polar environment. However, our results did not observe a significant shift of λ_{\max} in the purge drip by freezing-thawing, presumably indicating that the micro-environment of tryptophan residues in the sarcoplasmic proteins was not altered. In addition, the observation (Fig. 3) that frozen-thawed samples showed increased values of FI_{\max} as compared to fresh samples, are probably attributable to protein-protein association caused by freezing and thawing (Agyare & Damodaran, 2010; Sikorski, et al., 1976). Overall, from DSC and from fluorescence spectra we observed sarcoplasmic protein denaturation due to the freezing-thawing process, however, the freezing rate did not affect the observed denaturation. Therefore, we ascribe the difference in thaw loss between fast and slow freezing to denaturation of myofibrillar rather than sarcoplasmic proteins.

5. Conclusion

The freezing-thawing process increased the water loss of pork LTL muscle, and a further increase of thaw loss by around 28% was found at slow freezing as compared to fast freezing. Slow freezing caused a greater loss in the water-holding capacity of myofibrils and also increased surface hydrophobicity of myofibrils, indicating more pronounced myofibrillar protein denaturation in slow compared to fast freezing. DSC thermograms of muscle showed that freezing-thawing reduced the peak temperature and denaturation enthalpy for the first peak interpreted as myosin. The freezing-thawing process decreased the denaturation enthalpy and fluorescence intensity of purge drip, independent of freezing rate, suggesting some denaturation of sarcoplasmic proteins. The obtained data are in agreement with a presented model, which explains the mechanism of thaw loss based on the importance of myofibrillar protein denaturation in relation to the freezing rate.

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Figure captions:

Fig. 1. Center temperature curves to $-20\text{ }^{\circ}\text{C}$ of pork LTL steaks during fast-freezing (frozen at $-80\text{ }^{\circ}\text{C}$) and slow-freezing (frozen at $-20\text{ }^{\circ}\text{C}$). t_c : the characteristic freezing time for the temperature to decline from $-1\text{ }^{\circ}\text{C}$ to $-7\text{ }^{\circ}\text{C}$.

Fig. 2. Purge loss (%) (A), water-holding capacity (B) and surface hydrophobicity (C) of myofibrils in fresh and frozen-thawed pork steaks after fast and slow freezing. Purge loss of fresh pork steaks was measured at the period from 24 h to 48 h (defined as 24 h) and from 24 h to 72 h *post-mortem* (defined as 48 h). For frozen-thawed samples, purge loss including thaw loss was measured following thawing-storage at $2 \pm 1\text{ }^{\circ}\text{C}$ for 24 h and 48 h, respectively. BPB: bromophenol blue. Means \pm standard deviations are shown. a-d: Within each trait, mean values with the same letter do not differ ($P > 0.05$).

Fig. 3. Fluorescence spectra of purge drip from both fresh and frozen-thawed pork assigned to fast and slow freezing rate. The tryptophan maximum fluorescence intensity (FI_{\max}) is summarized in the table. a-c: mean values with the same letter do not differ ($P > 0.05$). SEM: Standard error of the mean.

Fig. 4. Model explaining how the freezing rate affect pH of the unfrozen water phase within the sarcomere. In slow freezing migration of water to the extracellular area cause transversal shrinkage of the sarcomere and a higher concentration of solutes and protons in the remaining liquid water. Consequently pH declines in the vicinity of myofibrillar protein surfaces, resulting in pronounced pH and ionic strength induced protein denaturation, leading to reduced water-holding and a high water loss upon thawing. In fast freezing small ice crystals are formed both intra- and extracellular, resulting in less

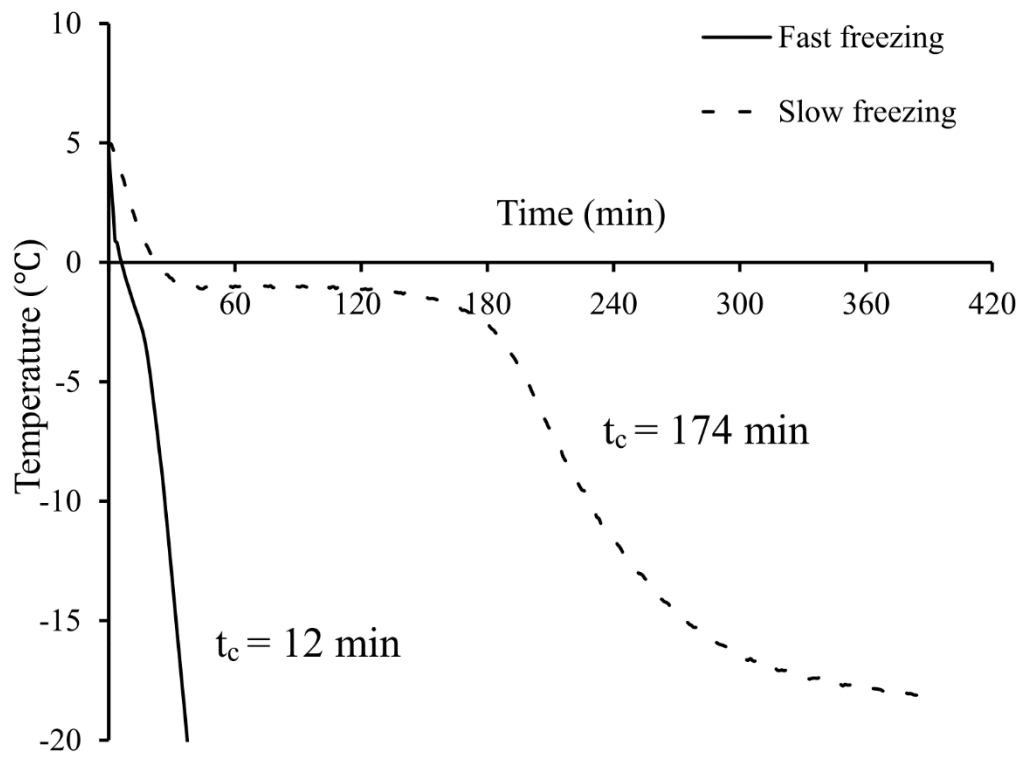
water migration, less transversal shrinkage, less reduction of pH in non-frozen water, less myofibrillar protein denaturation and hence less thaw loss as compared to slow freezing.

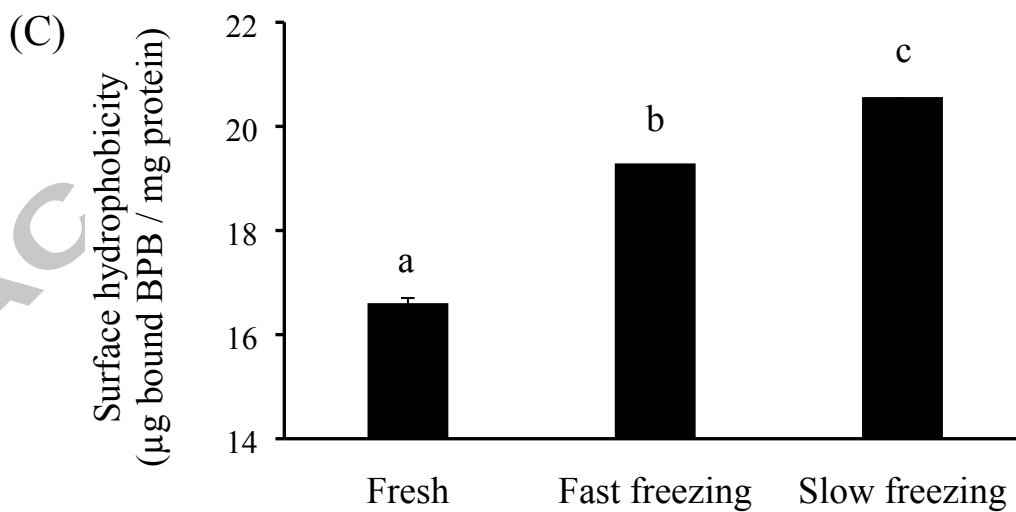
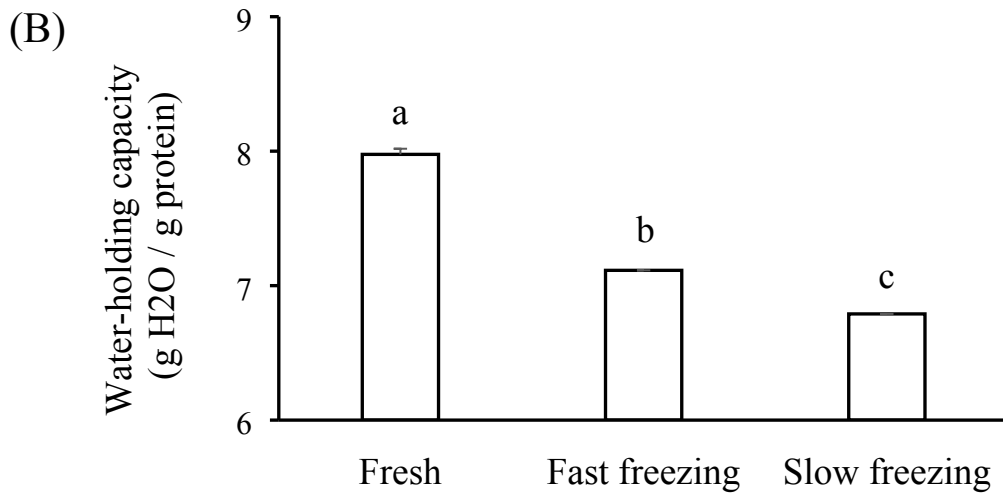
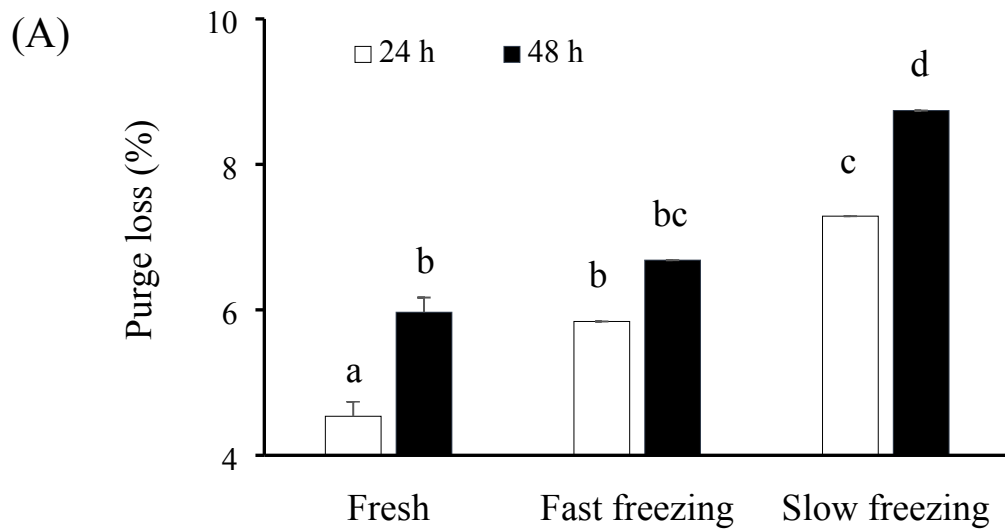
-H⁺: the more H⁺ in the diagram, the higher the concentration of protons within the sarcomere.

- : Ice crystals.

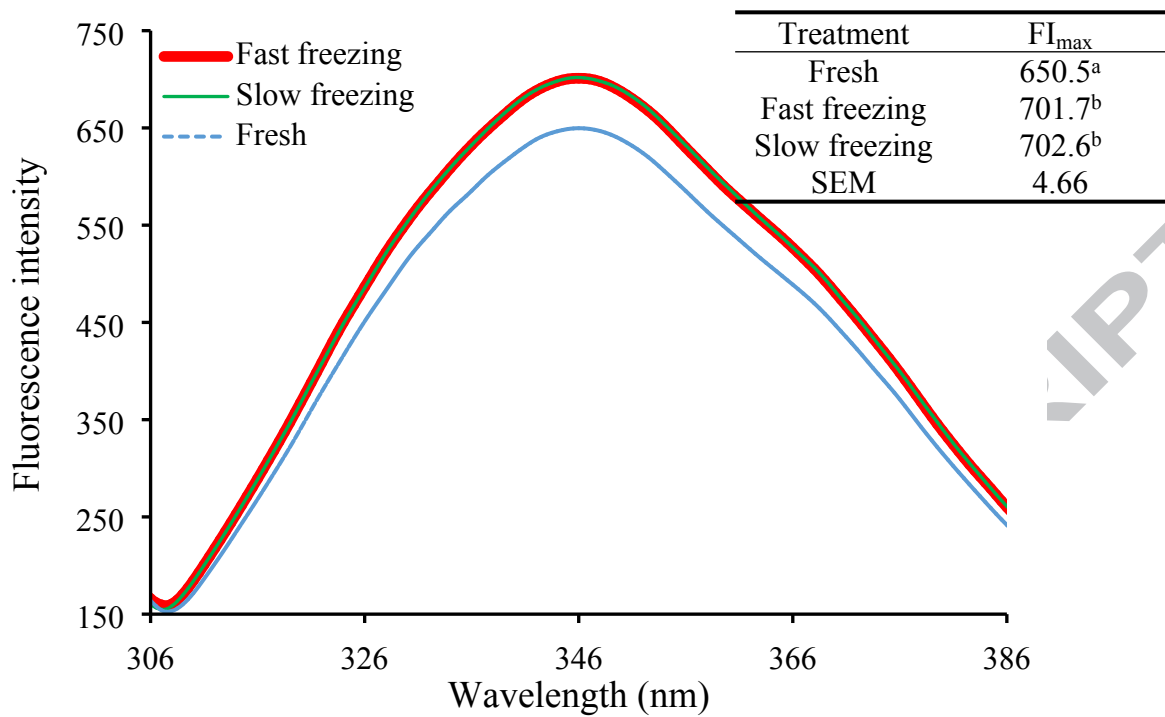
- : Ice crystals with a trapped H⁺.

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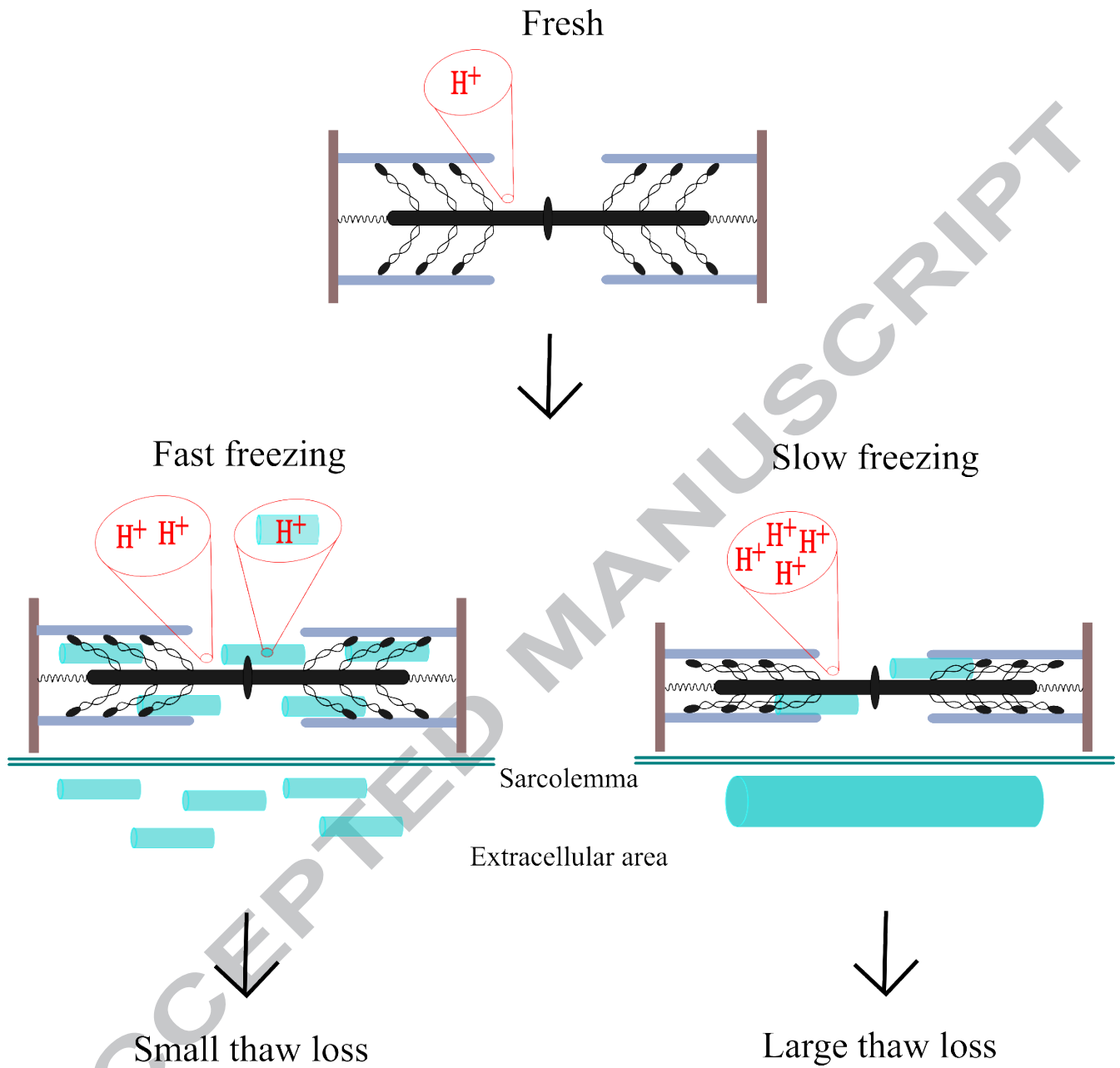


Table 1 Peak temperature (T_{peak}) and denaturation enthalpy (ΔH) in LTL muscle and purge drip from fresh and frozen-thawed pork steaks assigned to fast and slow freezing rate.

Treatment	Muscle						Purge drip	
	T_{peak1} (°C)	T_{peak2} (°C)	T_{peak3} (°C)	ΔH_1 (J g ⁻¹)	ΔH_2 (J g ⁻¹)	ΔH_3 (J g ⁻¹)	T_{peak} (°C)	ΔH (J g ⁻¹)
Fresh	53.4 ^a	64.6 ^a	77.8 ^a	1.54 ^a	1.53 ^a	1.31 ^a	65.5 ^a	7.00 ^a
Fast freezing	53.1 ^b	64.3 ^b	77.6 ^b	1.47 ^b	1.56 ^a	1.61 ^b	65.8 ^b	6.66 ^b
Slow freezing	53.0 ^b	64.4 ^b	77.6 ^b	1.42 ^b	1.39 ^b	1.62 ^b	65.7 ^b	6.62 ^b
SEM	0.05	0.04	0.03	0.02	0.03	0.03	0.05	0.08
Main effect	*	**	**	**	*	**	NS	*

SEM: Standard error of the mean. NS: not significant. * ($P < 0.05$), ** ($P < 0.01$). Within traits, superscripts with the same letter do not differ ($P > 0.05$).

Table 2 Water-holding capacity, surface hydrophobicity and particle size of myofibrils after exposure to combinations of different pH and KCl concentrations. The pH was measured in the myofibril pellets

pH of myofibrils	KCl concentration (M)	Water-holding capacity (g H ₂ O / g protein)	Surface hydrophobicity (µg bound BPB / mg protein)	Particle size (µm)			
				D(3,2)	D(4,3)	D(v,0.1)	D(v,0.9)
5.5	0.15	8.5 ^{ab}	18 ^a	33 ^a	120 ^a	12 ^a	262 ^a
5.4	0.42	9.0 ^b	19 ^a	52 ^{ab}	175 ^{ab}	22 ^{ab}	379 ^{ab}
5.3	0.69	8.9 ^b	20 ^{ab}	63 ^{abc}	199 ^{ab}	37 ^{abc}	423 ^b
5.2	0.96	6.8 ^{ac}	25 ^{bc}	66 ^{bc}	224 ^{bc}	35 ^{bc}	479 ^{bc}
5.1	1.13	6.4 ^c	27 ^c	86 ^c	291 ^c	53 ^c	608 ^{cd}
5.0	1.50	5.9 ^c	26 ^c	91 ^c	306 ^c	54 ^c	641 ^d
	SEM	0.3	1.0	2.5	7.2	2.1	14

during exposure.

SEM: Standard error of the mean. Within traits, superscripts with the same letter do not differ ($P > 0.05$).

Declaration of interests

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

- Slow freezing caused 28% greater thaw loss than fast freezing
- Water-holding capacity of myofibrils were 5% lower in slow than fast freezing
- Surface hydrophobicity of myofibrils were 6% larger in slow than fast freezing
- Freezing-thawing decreased the denaturation enthalpy values in meat and drip
- A model is proposed linking protein denaturation to the formation of thaw loss

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