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Myofibrillar protein characteristics of fast or slow frozen pork during subsequent storage at −3 °C

Yuemei Zhang [a], Arianna Magro [a,b], Eero Puolanne [a], Antonella Dalle Zotte [b], Per Ertbjerg [a,*]

[a] Department of Food and Nutrition, University of Helsinki, 00014 Helsinki, Finland
[b] Department of Animal Medicine, Production and Health, University of Padova, 35020 Legnaro, Padova, Italy

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ABSTRACT

This study aimed to investigate the effect of storage at −3 °C on myofibrillar protein in fast or slow frozen pork. Five pork loins at 48 h *post-mortem* were subjected to either fast (cold metal plate/−80 °C) or slow freezing (still air/−20 °C) followed by storage at −3 °C for 0, 1, 3, and 7 days before thawing. Freezing rate significantly influenced myofibrillar proteins within 3 days at −3 °C, evidenced by higher thaw loss, higher surface hydrophobicity and reduced water-holding of myofibrils, and accelerated appearance of a myosin-4 fragment (160 kDa) in slow freezing. However, these observed differences disappeared after 7 days of storage at −3 °C. The meat pH after thawing did not differ between fast and slow freezing rate. However, the pH values after thawing in both groups decreased with extended storage at −3 °C. Our results suggest that the beneficial effects of fast freezing are gradually lost by holding at −3 °C due to more extensive protein denaturation.

1. Introduction

Freezing and frozen storage have been extensively applied to extend shelf life of meat and meat products. However, frozen-thawed meat is well known to be of inferior meat quality to never-frozen meat considering some physiochemical deteriorations accompanied with thaw loss (Ballin & Lametsch, 2008; Coombs, Holman, Friend, & Hopkins, 2017). Myofibrillar proteins account for 60% to 70% of the total protein in the muscle structure, and the majority of water is trapped within the myofibrils. Any physiochemical and functional changes in myofibrillar proteins during freezing and subsequent storage could be associated with quality defects of meat in texture, flavour, and colour. Myofibrillar protein denaturation caused by freezing-thawing has been suggested due to observed greater surface hydrophobicity, lower water-holding capacity, and reduced denaturation enthalpy (Chan, Omana, & Betti, 2011; Wagner & Anõn, 1985).

The water content is around 75% in fresh lean meat. Some of the muscle water is strongly bound to proteins by hydrogen bonds. This water fraction has been defined as the bound water and it is strongly resistant to freezing, being not easily freezable at temperatures even below −40 °C (Aktas, Tulek, & Gokalp, 1997). The bulk water is freezable and has been reported to account for about 88% of total water in frozen pork (Xanthakis, Havet, Chevallier, Abadie, & Le-Bail, 2013), and thus contribute to drip formation during thawing (Offer & Knight, 1988). The majority of the bulk water, defined as the immobilized water, is held within the myofibrils. This water population, together with extracellular more loosely held water, is directly linked to water-holding capacity of meat influencing drip loss, cooking loss and thaw loss (Calvelo, 1981; Hamm, 1986). Freezing of meat usually starts in the extracellular area when the temperature declines until around −1.2 °C, whereas a lower freezing temperature of around −1.6 °C is required for intracellular ice formation (Hamm, 1986).

The water in the muscle structure is progressively turning into ice crystals as the temperature is reduced, however, a fraction of the water still remains unfrozen (Calvelo, 1981; Li, Chinachoti, Wang, Hallberg, & Sun, 2008). It has been suggested by Xiong (1997) that protein denaturation is induced by the presence of concentrating solutes in the remaining unfrozen water as freezing progresses. Our previous studies (Zhang & Ertbjerg, 2019; Zhang, Puolanne, & Ertbjerg, 2021) observed irreversible decreased water-holding capacity of myofibrils following temporary exposure to decreased pH combined with concentrating solutes, and thus proposed the potential involvement of decreased pH in the unfrozen water in causing protein denaturation during freezing. The importance of pH for the water-holding capacity within the myofibrils has been emphasized in earlier studies (Hamm, 1986; Offer & Knight, 1988), and a shift of pH towards the isoelectric point of myofibrillar

* Corresponding author.
E-mail address: per.ertbjerg@helsinki.fi (P. Ertbjerg).

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proteins (close to pH 5.0) leads to reduced net charge within the myofilaments (Hamm, 1986). Electrostatic repulsive forces between and within the charged filaments would then be expected to be reduced, causing myofibrillar shrinkage and as a result, decreased water-holding within the myofibrils as reviewed by Puolanne and Halonen (2010) and Offer and Knight (1988). Cheng et al. (2019) has observed lower pH in frozen-thawed beef than prior to freezing. Also Ali et al. (2015) has found lower pH in chicken breast after freeze-thaw cycles. However, agreement in scientific literature on the changes of pH in freezing-thawing is still lacking, and some studies found no pH difference between non-frozen and frozen-thawed meat (Kim, Meyers, Kim, Liceaga, & Lemenager, 2017; Medić et al., 2018).

Several studies have over the years proposed that the rate of protein denaturation is highly related to the subzero temperature (Buttkus, 1970; Kaale & Eikevik, 2016), and the maximum rate of protein denaturation reaction usually exists at a temperature just below the freezing point (Duun & Rustad, 2008; Love & Elerian, 1964). The concentrating solutes and decreased pH in the unfrozen water phase brought about by a reduction in temperature and crystallization of water (approximately 40–70%), are hypothetically responsible for the protein denaturation occurring within this temperature range (Calvelo, 1981). A further reduction of temperature during freezing, however, could potentially have a counteractive effect and therefore reduce the rate of protein denaturation (Li et al., 2018; Love & Elerian, 1964). Additionally, the activity of many enzymes remains significant in this partially frozen system, when compared to deep freezing, which in turn may accelerate the enzymatic reactions and therefore lead to additional protein deterioration (Behnke, Fennema, & Cassens, 1973; Ge, Xu, & Xia, 2015). Meat is preferably frozen to be preserved for a long time in the industry. It is well known that the freezing rate could strongly influence the extent of quality deterioration in frozen/thawed meat, and fast frozen meat has superior myofibrillar protein characteristics and lower thaw loss compared to slow frozen (Kim, Kim, Seo, Setyabrata, & Kim, 2018; Wagner & Anõn, 1985; Zhang & Ertbjerg, 2019). When frozen meat is thawed in the industry, it will take time to be completely thawed. For example, beef carcasses in a cold chamber of around 5 °C with a relative low air circulation of 0.2 m/s may take four to five days to thaw. This causes a high probability that the temperature of meat during industrial thawing will be around −3 °C for a longer period of time, due to the high latent heat around −3 °C (Rahman, Kasapis, Guizani, & Al-Amri, 2003). Even in some modern systems, i.e. steam thawing and microwave thawing, the defrosting is stopped at around −4 to −2 °C in order to avoid thaw loss in thawing room or hot spot formation in meat during microwave thawing. Hence it would be reasonable to investigate the effect of the industrial thawing-storage on myofibrillar protein characteristics in frozen meat with different freezing rates.

Porcine *Longissimus thoracis et lumborum* (LTL) muscle was subjected to either fast freezing at −80 °C or slow freezing at −20 °C followed by subzero temperature treatment at −3 °C for 0, 1, 3 and 7 days prior to thawing at 2 ± 1 °C for 16 h. Myofibrillar protein denaturation was evaluated by measuring water-holding capacity, surface hydrophobicity and SDS-PAGE profile analysis of myofibrils, and thaw loss and pH of meat samples. Results of this study could be utilized by industry to develop optimized freezing/thawing regimes to preserve meat quality.

2. Materials and methods

2.1. Sample processing

Five pork loins (LTL) were obtained at 48 h *post-mortem* from five different pigs slaughtered at HKScan Ltd. slaughterhouse (Forssa, Finland). The visible connective tissue and fat were trimmed off and then the LTL muscle was isolated and sliced into 18 cuts along the muscle length at 48 h *post-mortem*. The average pH was 5.5 ± 0.1 measured using a portable pH meter PHM201 (Radiometer Analytical SAS, Lyon, France). L*, a* and b* average values were 53.4 ± 3.7, 7.5 ± 1.2 and 5.0 ± 1.1, respectively, measured using a Minolta Chroma meter CR-400 (Minolta Camera Co.Ltd., Osaka, Japan) with a 8 mm diameter of aperture following calibration by a white tile (C: Y = 93.6, x = 0.3130, y = 0.3193). Each cut (around 9 × 6 × 1.5 cm) was weighed and packaged under vacuum (Boss Vakuum, Bad Homburg, Germany). Two cuts from each of five muscles were randomly collected as fresh samples, and the remaining cuts were thereafter randomly distributed to a 2 × 4 factorial combinations (two cuts for each combination) with two freezing rates (fast and slow freezing) and four subsequent storage periods at −3 °C (0, 1, 3, 7 days). The fresh samples ($n = 2 \times 5$) were stored for 24 h at 2 ± 1 °C. Fast frozen samples were placed in a − 80 °C freezer (Ultra Low, SANYO, Japan) on a cold metal plate and slow frozen samples were placed in a − 18 °C walk-in freezer (Huurre, Vantaa, Finland). Both freezing systems used still air and the meat samples were frozen in a single layer. After 24 h, slow frozen samples were moved into the freezer of −80 °C in order to diminish any effects of time and temperature during frozen storage before analysis. The internal temperature for meat samples during freezing were monitored by type K thermocouples connected to a data logger (Honeywell DPR-3000). The characteristic freezing times for fast and slow frozen samples were around 12 min and 174 min, respectively, as previously described (Zhang & Ertbjerg, 2019). The fast and slow frozen samples were then moved into a bench-top-type controlled temperature chamber equipped with cooling and heating units (Espec Corp., Osaka, Japan) with a setting subzero temperature of −3 ± 0.5 °C. After being stored for around 6 h to reach the target temperature, meat cuts were kept at −3 °C for 0, 1, 3, and 7 days, respectively, followed by thawing overnight at 2 ± 1 °C in a cold room. The frozen-thawed pieces following the assigned treatment were sampled for pH and thaw loss, and myofibrils for each group were isolated for the determination of surface hydrophobicity, water-holding capacity, and SDS-PAGE analysis.

2.2. The pH and thaw loss

For the determination of pH, about 5 g meat was homogenized in 25 mL distilled water, and the pH value of the homogenate was measured by an insertion probe electrode (Mettler-Toledo Inlab 427) at room temperature. Following treatment, the vacuum bags were opened and the meat samples were blotted dry using filter paper. For the calculation of thaw loss, the meat weight after each assigned treatment was compared with the initial weight taken at 48 h *post-mortem*.

2.3. Isolation of myofibrils

Meat samples (around 4 g) in 20 mL of cold MES buffer (75 mM KCl, 20 mM MES, 2 mM $MgCl_2$, 2 mM EGTA, pH 5.5) were homogenized for 10 s at 13,500 rpm using an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany), followed by centrifuging at 2400 ×*g* at 4 °C for 10 min. The supernatant was then discarded, and the pellet was washed twice with the same procedure to remove soluble proteins. Myofibrils in the final pellet were resuspended in MES buffer and then used for further analysis.

2.4. Surface hydrophobicity of myofibrils

Surface hydrophobicity of the pellets was determined following the method of Zhang and Ertbjerg (2019). The pellet was re-suspended in MES buffer and the protein content was then analyzed using DC Protein Assay Kit. To 1 mL of protein suspension (adjusted to 2 mg/mL), 80 μL of 1 mg/mL bromophenol blue (BPB) was added, and then the mixture was incubated for 10 min at room temperature and centrifuged at 10,000 ×*g* for 3 min. The collected supernatant was diluted 10 times and the absorbance at 595 nm was then measured. The surface hydrophobicity was then calculated as the amount of BPB bound per mg protein.

2.5. Water-holding capacity of myofibrils

One gram of myofibril pellets was resuspended in the MES buffer (9 mL) by homogenizing for 30 s at 9500 rpm. Water-holding capacity of isolated myofibrils was measured following our previous method (Zhang & Ertbjerg, 2018) and was then calculated as the amount of water held per g protein.

2.6. SDS-PAGE

Myofibrillar protein suspension for SDS-PAGE analysis was prepared as for the determination of surface hydrophobicity and then diluted to the same protein concentration. Subsequently, the diluted protein suspension was mixed with NuPAGE™ LDS Sample Buffer (4 ×) and NuPAGE™ Sample Reducing Agent (10 ×) (Invitrogen, Carlsbad, CA) and distilled water, resulting in a protein concentration in the mixture of around 1.5 mg/mL. The mixture was heated at 70 °C for 10 min and 18 μg protein in each well was then loaded onto NuPAGE™ Novex 3–8% Tris-Acetate gels. The electrophoresis was run at 150 V for 50 min. Coomassie brilliant blue R250 dye was used to stain the gel and then a digital camera was used to capture the image from the gel after destaining. Representative samples from each treatment were selected for qualitative analysis.

2.7. Statistical analysis

Data were analyzed by using general linear model in the IBM SPSS Statistics 25 software. Each loin from different animals, was represented at every treatment and storage period resulting in 5 replicates per measurement. For each of the five muscles, duplicates were done for thaw loss, and triplicates were done for pH measurement, surface hydrophobicity of myofibrils, water-holding capacity of myofibrils, and SDS-PAGE analysis. A completely randomized design with two freezing rates (fast and slow freezing) and four subzero temperature storage durations (0, 1, 3 and 7 days) was used. Freezing rate, subzero temperature storage durations, and their interaction were arranged as fixed factors, and muscle number was regarded as a random factor. Bonferroni test was used to evaluate significant differences with $P < 0.05$ between means of each group.

3. Results

3.1. Thaw loss

Freezing rate and subsequent subzero temperature storage both significantly affected thaw loss of frozen-thawed pork cuts (Fig. 1 and Table 1). The slow freezing sample had greater ($P < 0.05$) thaw loss compared to fast freezing samples when stored at −3 °C for 3 days followed by thawing at 2 °C overnight, but the difference decreased with storage time and was no longer significant ($P > 0.05$) after 7 days of subzero temperature storage.

3.2. pH

A significant effect of subsequent subzero temperature storage on pH was observed in both fast and slow freezing samples (Fig. 2 and Table 1). The pH values were 5.47 and 5.46 on day 0 in fast and slow freezing samples, respectively, and thereafter decreased ($P < 0.05$) to 5.39 and 5.40 within one week of storage at −3 °C. However, the pH was unaffected ($P > 0.05$) by the freezing rate throughout the storage period.

3.3. Surface hydrophobicity of myofibrils

As shown in Fig. 3 and Table 1, an increasing effect ($P < 0.05$) of subsequent subzero temperature storage on surface hydrophobicity of myofibrils in both fast and slow freezing groups was observed, where

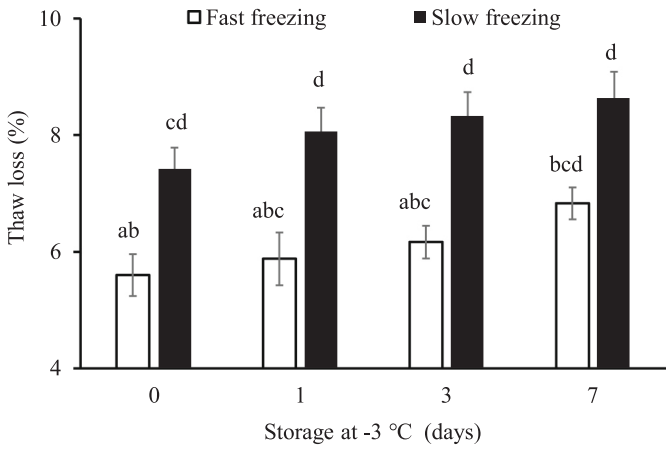

Fig. 1. The effect of subsequent subzero temperature storage on the thaw loss of fast and slow frozen pork cuts. Frozen cuts were firstly stored at −3 °C for 6 h to reach the temperature, and then were kept at −3 °C for 0, 1, 3, and 7 days, respectively, followed by thawing overnight at 2 ± 1 °C. Means ± standard errors are shown. a-d: Different letters indicate significant difference ($P < 0.05$).

Table 1
P values of freezing effect (fast vs slow freezing rate), subsequent subzero temperature storage at −3 °C and their interaction on thaw loss, pH, water-holding capacity of myofibrils, and surface hydrophobicity of myofibrils.

Effects	Thaw loss	pH	Water-holding of myofibrils	Surface hydrophobicity
Freezing	0.00**	0.57	0.00**	0.07
Subzero temperature storage	0.05*	0.02*	0.02*	0.00**
Freezing × Subzero temperature storage	0.85	0.44	0.16	0.36

* $P < 0.05$.
** $P < 0.01$.

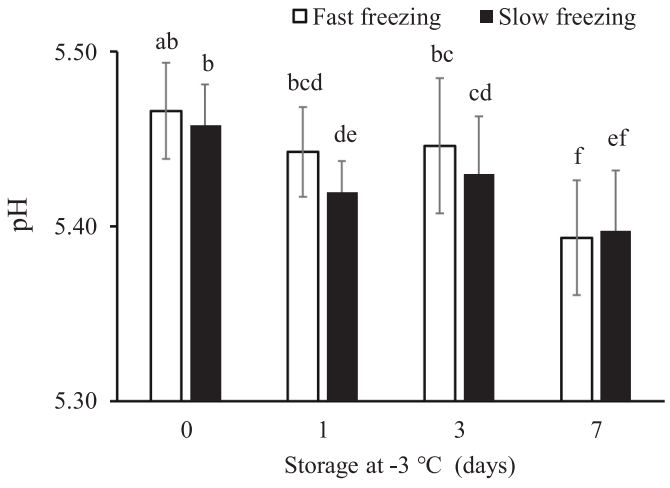

Fig. 2. The effect of subsequent subzero temperature storage on the pH of fast and slow frozen pork cuts. Frozen cuts were firstly stored at −3 °C for 6 h to reach the temperature, and then were kept at −3 °C for 0, 1, 3, and 7 days, respectively, followed by thawing overnight at 2 ± 1 °C. Means ± standard errors are shown. a-f: Different letters indicate significant difference ($P < 0.05$).

fast and slow freezing samples increased significantly by around 15% and 10%, respectively, after 7 days of storage. Slow freezing had larger ($P < 0.05$) values of surface hydrophobicity compared to fast freezing for samples stored at −3 °C for 0 or 1 day before thawing, but this increasing

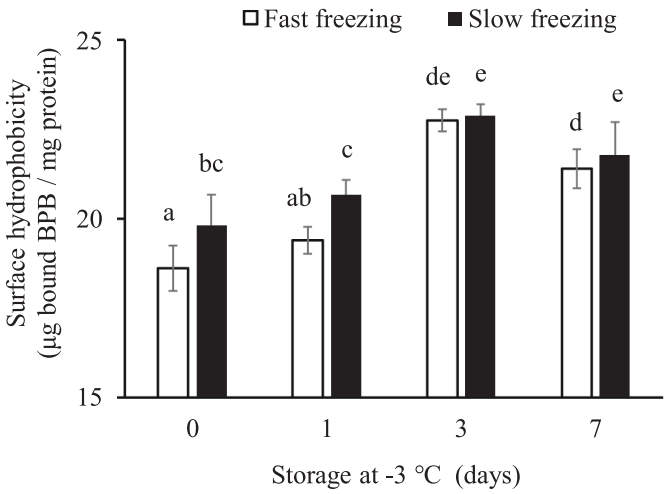

Fig. 3. The effect of subsequent subzero temperature storage on the surface hydrophobicity of myofibrils isolated from fast and slow frozen pork cuts. Frozen cuts were firstly stored at −3 °C for 6 h to reach the temperature, and then were kept at −3 °C for 0, 1, 3, and 7 days, respectively, followed by thawing overnight at 2 ± 1 °C. Means ± standard errors are shown. a-e: Different letters indicate significant difference ($P < 0.05$).

effect disappeared ($P > 0.05$) at the longer times of 3 and 7 days.

3.4. Water-holding capacity of myofibrils

The water-holding capacity of myofibrils showed an opposite trend compared to surface hydrophobicity and decreased ($P < 0.05$) with increased subzero temperature storage time independent of the freezing rate (Fig. 4). Myofibrils isolated from fast freezing samples showed higher values ($P < 0.05$) of water-holding capacity compared to that of slow freezing samples within the first 3 days of storage, but no difference ($P > 0.05$) was observed for samples stored at −3 °C for 7 days.

3.5. SDS-PAGE

Fig. 5 shows a representative SDS-PAGE gel image. A protein band of around 160 kDa was observed in frozen-thawed samples following storage at −3 °C. This band has been recognized as a protein fragment originating from myosin-4 (MYH4) in our previous study (Zhang et al., 2021). In slow frozen samples this band was visible on day 0 (without storage), whereas the same band appeared in the fast frozen samples not earlier than at 1 day of storage.

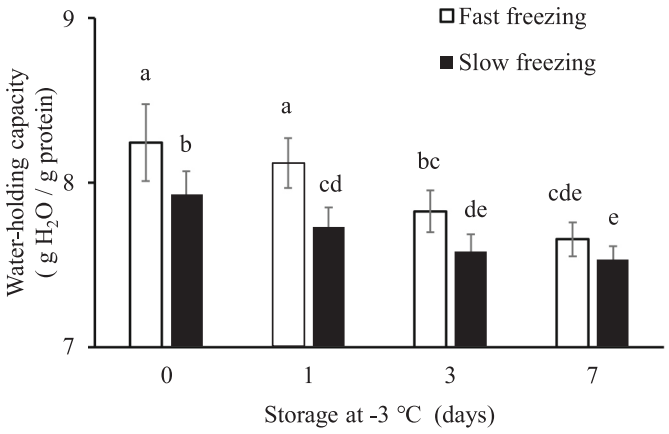

Fig. 4. The effect of subsequent subzero temperature storage on the water-holding capacity of myofibrils isolated from fast and slow frozen pork cuts. Frozen cuts were firstly stored at −3 °C for 6 h to reach the temperature, and then were kept at −3 °C for 0, 1, 3, and 7 days, respectively, followed by thawing overnight at 2 ± 1 °C. Means ± standard errors are shown. a–e: Different letters indicate significant difference ($P < 0.05$).

4. Discussion

This is the first study reporting an effect of storage at −3 °C on myofibrillar proteins in frozen pork muscle following fast or slow freezing in order to study conditions where meat may stay at around −3 °C for a length of time of relevance to industrial thawing and subsequent storage. Fast freezing showed a lower amount of thaw loss and less detrimental effects on the myofibrillar protein attributes compared to slow freezing at the initial phase of storage at −3 °C, as indicated by the observations of the lower values of surface hydrophobicity (Fig. 3), greater water-holding (Fig. 4), and delayed fragmentation of myosin (Fig. 5) in fast compared to slow freezing. However, increased storage time at −3 °C showed detrimental effects on myofibrillar proteins at both fast and slow freezing rates, and the initial differences disappeared within one week of storage. Our results thus suggest that storage at −3 °C following freezing at −20 °C or lower results in more excessive denaturation of myofibrillar proteins, which diminish the effects of freezing rate on meat quality as confirmed by the observation that the difference of thaw loss between fast and slow frozen groups disappeared with increased storage time at −3 °C (Fig. 1).

The existence of a subzero temperature at which there is a maximum rate of protein denaturation reaction has been proposed in earlier studies. This temperature has been recognized by Calvelo (1981) as −3 °C in *post-rigor* beef. In agreement, Behnke et al. (1973) found that rates of ATP consumption and lactate accumulation were faster when *pre-rigor* frozen beef and poultry muscles were kept at −3 °C, compared to other subzero temperatures. Duun, Hemmingsen, Haugland, and Rustad (2008) observed lower water-holding capacity in pork when stored at −2 °C as compared to chilled storage at 3.5 °C and frozen storage at −36 °C. These changes were attributed to the decreasing temperature and concentrating inorganic salts in the unfrozen water outside of the ice crystals in freezing. The potential role of reduced pH in the unfrozen water has been proposed to cause protein denaturation during freezing (Zhang & Ertbjerg, 2019). In fast freezing fast growing small ice crystals might trap protons and therefore cause less decline of pH in the non-frozen water and induce less myofibrillar protein denaturation during freezing-thawing as compared to slow freezing. This could offer an explanation of why we observed higher water-holding and lower surface hydrophobicity of the washed myofibrils in fast compared to slow freezing. Tuell, Seo, and Kim (2020) and Kim et al. (2018) have recently reported higher water-holding capacity and better myofibrillar protein properties in fast frozen pork muscle compared to slow. The pH of fast frozen-thawed meat was not significantly different ($P > 0.05$) compared to that of slow frozen-thawed meat (Fig. 2), since the condition of reduced pH in the unfrozen water in freezing is believed as reversible as the melting of ice crystals is accompanied with the dilution of the solutes in the unfrozen water during thawing. However, decreased pH ($P < 0.05$) of thawed meat were observed in both fast and slow deep frozen pork after storage at −3 °C for 7 days. In agreement, decreased pH has been found by Lan, Shang, Song, and Dong (2016) in rabbit hind leg muscle and by Ding et al. (2020) in pork loins after being stored at around −3 °C one week. However, several studies on freezing did not find any influence on the changes of pH in frozen-thawed beef as compared to fresh (da Silva Bernardo et al., 2020; Kim et al., 2017). In storage at −3 °C, most of muscle water has been frozen, but there is still a fraction of unfrozen water within the muscle, including the water that is more tightly attached to proteins. Solutes and protons would become more concentrated in the unfrozen water phase outside the ice crystals, and therefore a decline of pH is to be expected within the myofibrils. Exposure to low pH and high ionic strength would then cause denaturation of myofibrillar proteins (Zhang et al., 2021) occurring at −3 °C and a subsequent release of hydrogen ions after thawing (Leygonie, Britz, &

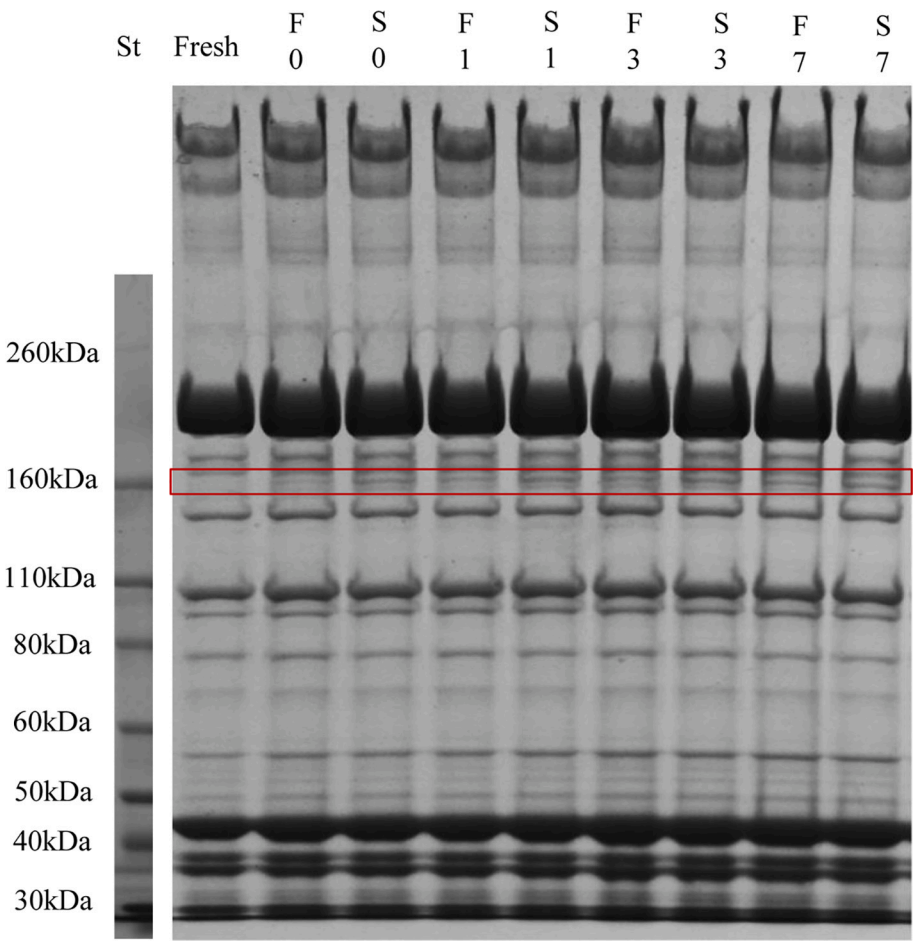

Fig. 5. Representative SDS-PAGE of myofibrils collected from fast and slow frozen-thawed pork cuts following subsequent subzero temperature storage. Frozen cuts after fast or slow freezing were firstly stored at −3 °C for 6 h to reach the temperature, and were then kept at −3 °C for 0, 1, 3, and 7 days, respectively, followed by thawing overnight at 2 ± 1 °C. Fresh represents the 72 h *post-mortem* pork cut. F and S represent fast and slow freezing, respectively. The numbers of 0, 1, 3, 7 in the figure represent different subzero temperature storage days. St is molecular weight standard.

Hoffman, 2012). Together with the effects of the maximum denaturation at −3 °C this might explain why more accelerated myofibrillar protein deterioration was observed in fast frozen pork compared to that in slow frozen pork when subsequently stored at −3 °C for prolonged time, as confirmed by the similar myofibrillar protein attributes observed between fast and slow freezing samples after one week of storage.

Water-holding capacity of myofibrils has been used to estimate denaturation of myofibrillar proteins occurring during freezing and thawing in our previous studies (Zhang et al., 2021; Zhang & Ertbjerg, 2018, 2019). The majority of myowater in the meat tissue is trapped within the myofibrils, and therefore any changes in the amount of myowater could potentially be related to the physicochemical changes of myofibrillar proteins. An effect of freezing rate on water-holding capacity of myofibrils was not observed after 7 days of storage at −3 °C, and interestingly, the fast frozen group developed similar values of thaw loss as the slow frozen group. This suggests a major role of myofibrillar protein denaturation in freezing-thawing in causing increased amount of thaw loss. It is well known that ultimate pH has a great influence on the water-holding of meat (Hamm, 1986). The observation that the pH value decreased by more than 0.05 units in both fast and slow frozen pork after being stored at −3 °C for 7 days (Fig. 2) would then be expected to negatively affect the water-holding to some extent, thereby leading to a decrease of water-holding in both fast and slow frozen groups after prolonged storage. Freezing and thawing have been reported to cause increased degradation of myofibrillar proteins in pork (Liu, Xiong, & Rentfrow, 2011; Pomponio, Bukh, & Ruiz-Carrascal, 2018) and beef (Aroeira et al., 2020; Grayson, King, Shackelford, Koohmaraie, & Wheeler, 2014; Setyabrata & Kim, 2019). In low pH beef Ertbjerg, Mielche, Larsen, and Moller (1999) observed release of lysosomal cathepsins followed by myosin degradation and a parallel increase of a myosin degradation product of 150–160 kDa as observed in the present study. In addition, increased activation of calpain-2 after freezing and thawing has been observed by Colle et al. (2018) in beef and Zhang and Ertbjerg (2018) in pork. Taken together, these results suggest that freezing-thawing results in an increased proteolytic potential. The myosin-4 fragment occurring in the frozen-thawed group (Fig. 5) is a product of fragmentation of the myosin molecule (Zhang et al., 2021). Also increased ionic strength and pH decline as hypothesized to occur in the unfrozen water during freezing (Zhang et al., 2021) would then disrupt the inner structure of myosin filaments promoting the conformational changes within myosin filaments and consequently dissolution and denaturation. Slow freezing often results in a larger mechanical damage to muscle fibers as observed by Kim et al. (2018) and Zhang, Niu, Chen, Xia, and Kong (2018) in slow frozen-thawed pork compared to fast frozen. Also, more pronounced pH decline and ionic strength increase in slow freezing would induce more pronounced protein denaturation as compared to the condition of fast freezing (Zhang & Ertbjerg, 2019). This could possibly explain the observation in this study that the myosin-4 fragment appeared earlier in slow freezing compared to fast freezing in the initial phase of storage at −3 °C. However, the effect of the freezing rate on the myosin-4 fragment was not evident on day 7, which is likely attributable to increased proteolytic degradation or pH and ionic strength induced protein denaturation when deep frozen samples were subsequently stored at a temperature below the freezing point for extended storage time (Ge et al., 2015; Pomponio et al., 2018). Zeng, Li, and Ertbjerg (2017) observed improved water-holding associated with increased proteolytic degradation within the myofibrils isolated from pork muscle. However, the appearance of myosin-4 fragment in this study seems not to positively affect water-holding capacity within the isolated myofibrils. We speculate that protein denaturation

occurring in freezing-thawing could more than counterbalance the effect of proteolysis on water-holding, thereby leading to the observed decrease of water-holding capacity within the myofibrils during subsequent storage at −3 °C.

Surface hydrophobicity has been used to indicate protein denaturation in pork muscle (Chelh, Gatellier, & Santé-Lhoutellier, 2006; Liu, Arner, Puolanne, & Ertbjerg, 2016), and protein unfolding and aggregation could affect the amount of the hydrophobic residues exposed on the protein surface and consequently change the observed values of surface hydrophobicity. The subsequent storage at −3 °C in this study accelerated the increase of surface hydrophobicity in fast frozen pork and the difference between fast and slow freezing groups was not detectable on day 3 and 7. We speculate that the changes of surface hydrophobicity in fast and slow frozen pork with prolonged storage at −3 °C may be a consequence of the balance between unfolding and aggregation of myofibrillar proteins induced by the initial ice crystal formation during freezing (Calvelo, 1981) and later on by the melting of crystals affecting pH and ionic strength of the non-frozen water during the process (Zhang et al., 2021).

5. Conclusion

This study observed higher thaw loss, lower water-holding of myofibrils and greater surface hydrophobicity in slow compared to fast freezing within 3 days of storage at −3 °C. Also, SDS-PAGE of isolated myofibrils showed earlier appearance of a myosin-4 fragment (around 160 kDa) in slow compared to fast frozen pork. The subsequent storage at −3 °C accelerated the increase of surface hydrophobicity and decrease of water-holding of the isolated myofibrils with prolonged storage, and as a result, the final levels of these different parameters reached similar values when fast and slow frozen samples were kept at −3 °C for 7 days. Overall, these observations indicate that more excessive protein denaturation occurred in the myofibrillar fraction with extended storage time at −3 °C. The freezing rate did not affect the pH of meat after thawing, whereas prolonged storage at −3 °C resulted in the decrease of pH in both fast and slow frozen pork observed on day 7. This study shows that prolonged storage of meat at a temperature of −3 °C diminish the positive effects of the fast freezing on myofibrillar protein attributes. Therefore, it is recommended that the meat industry adjusts the thawing process in order to shorten the time for meat having the temperature of −3 °C as much as possible during thawing.

Authorship statement

Conception and design of study: All authors.

Acquisition of data: Yuemei Zhang, Arianna Magro.

Analysis and/or interpretation of data: Yuemei Zhang, Eero Puolanne, Per Ertbjerg.

Drafting the manuscript: Yuemei Zhang, Eero Puolanne, Antonella Dalle Zotte, Per Ertbjerg.

Revising the manuscript critically for important intellectual content: Yuemei Zhang, Eero Puolanne, Antonella Dalle Zotte, Per Ertbjerg.

Approval of the version of the manuscript to be published: All authors.

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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