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Mimicking myofibrillar protein denaturation in frozen-thawed meat: Effect of pH at high ionic strength

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Abstract

This study aims at providing new insight on protein denaturation in freezing-thawing. Freezing-thawing minced pork reduced water-holding of myofibrils and increased surface hydrophobicity. One additional freezing-thawing cycle at slow freezing rate caused appearance of a 160 kDa myosin-4 fragment in SDS-PAGE, further decreased water-holding of myofibrils and increased surface hydrophobicity. Fresh minced pork was exposed to either high salt (2 M KCl) only or high salt with lower pH to mimic conditions in freezing. Exposure to high salt only increased water-holding of myofibrils and hence did not reproduce myofibrillar protein changes in freezing. Exposure to combinations of lower pHs and high salt decreased water-holding and increased surface hydrophobicity, suggesting myofibrillar protein denaturation occurred by a comparable mechanism as in freezing-thawing. We propose that exposure to decreased pH combined with high solute concentrations in the unfrozen water of frozen meat is the primary cause of myofibrillar protein denaturation in frozen-thawed meat.

Keywords: Freezing, Water-holding capacity, Myofibril structure, Particle size, Myosin, Surface hydrophobicity

1. Introduction

Freezing has traditionally been used as the preferred method to preserve meat and meat products. However, some quality deteriorations, particularly water loss accompanied with a loss of valuable nutrients, could occur associated with freezing-thawing and repeated freezing-thawing cycles (Leygonie, Britz, & Hoffman, 2012). Some detrimental physiochemical changes in repeated freezing-thawing of pork and beef such as decreased water-holding and accelerated protein denaturation have been reported (Cheng et al., 2019; Zhang, Li, Diao, Kong, & Xia, 2017). Freezing rate has a strong influence on thaw loss, and the size and distribution of ice crystals inside and outside the muscle fibers are generally believed to be the main driving force (Anõn & Calvelo, 1980; Farouk, Wieliczko, & Merts, 2003; Hamm, 1986). However, a role of protein denaturation in the formation of thaw loss and how it is affected by the freezing rate has been recognized by Petrović, Grujić, & Petrović (1993), Wagner & Anõn (1986) and Zhang & Ertbjerg (2019), showing that slow freezing causes more pronounced myofibrillar protein denaturation and a resultant larger thaw loss as compared to fast freezing. Knowledge on the effect of repeated freezing and thawing cycles in relation to the freezing rate on protein denaturation is still lacking.

Xiong (1997) suggested that changes in the water phase during freezing can lead to alterations in the chemical and physical environment of the muscle proteins which will contribute to protein denaturation. Freezing-induced denaturation of protein has traditionally been attributed to the increase in solutes concentration in the unfrozen water phase of the muscle structure (Shenouda, 1980; Sikorski, Olley, Kostuch, & Olcott, 1976). Crystallization of a high percentage of water in the muscle structure takes place during freezing, being approximately 90% at -20 °C, however, a fraction of the water remains unfrozen (Li, Chinachoti, Wang, Hallberg, & Sun, 2008). As freezing progresses, muscle proteins will be exposed to progressively concentrating solutes in the remaining unfrozen phase, and this process could

lead to modifications of the protein structure and therefore protein denaturation (Shenouda, 1980). Conformational changes of myosin has been reported in salmon muscle after being exposed to ionic strength above 1.5 M (Lin & Park, 1998). Puolanne & Halonen (2010) suggested that selective binding of chloride ions to the hydrophobic areas of myosin filaments affect water-holding. The addition of 1 to 1.5 M sodium chloride to a meat system corresponds to a relative high ionic strength and results in a maximal increase of water-holding capacity (Hamm, 1986). Salting before freezing has by Jiang, Nakazawa, Hu, Osako, & Okazaki (2019) been reported to improve water-holding and texture properties following multiple freezing-thawing cycles. As the interactions of myofibrillar proteins and water influence water-holding, the overall myofibrillar protein denaturation that occurs during freezing potentially contributes to a decrease in water-holding capacity (Zhang & Ertbjerg, 2019). Hence it can be questioned if the mechanism of protein denaturation in freezing is related to a direct effect of the increased ionic strength in the unfrozen water.

Puolanne & Halonen (2010) reviewed that pH and ionic strength can greatly affect water-holding capacity in meat systems via their interactions with proteins and water. This has been associated with changes of the electrostatic repulsive force in and between the charged myofilaments caused by pH and ionic strength (Hamm, 1986; Offer & Knight, 1988). Puolanne and Peltonen (2013) found that changing the pH from 5.7 to 5.0 in meat had a much larger effect on water-holding than changing the ionic strength from 0.5 to 1.5. The observation that below -20°C more than 90% of the water will freeze out (Calvelo, 1981), should theoretically lead to roughly a > 10-fold increase of ionic strength and proton concentration in the unfrozen water phase during freezing of meat. Puolanne and Kivikari (2000) found that the buffering capacity increases with added salt especially at low pH values. The buffering capacity of

myofibrillar proteins would therefore absorb part of the protons accumulating in concentrating solutes in the unfrozen water during freezing. Our previous study observed detrimental protein changes in myofibrils following exposure to a combination of lower pH (around 5.2) and higher KCl concentration (around 1 M), with the altered properties being comparable to that observed in frozen-thawed meat (Zhang & Ertbjerg, 2019).

The aim in the present study was to mimic denaturation of myofibrillar proteins in frozen-thawed meat by exposure of fresh minced meat to lower pH levels at high ionic strength. To obtain information on the combined effect of low pH and high ionic strength, we here exposed fresh minced pork *longissimus thoracis et lumborum* (LTL) muscle to a high ionic strength (corresponding to 2 M KCl) as occur during freezing (Finn, 1932; Offer & Knight, 1988; Ohta & Tanaka, 1978), combined with a lower pH (from 5.5 to 5.2) as hypothesized to occur during freezing (Zhang & Ertbjerg, 2019), and we studied the effect of the exposure on myofibrillar proteins characteristics as measured by water-holding capacity, surface hydrophobicity and SDS-PAGE profile of myofibrils. The present study further focused on increased effects of freezing and thawing cycles in relation to the freezing rate on the myofibrillar protein denaturation in pork by measuring water-holding capacity, surface hydrophobicity, particle size and SDS-PAGE profile of myofibrils.

2. Materials and methods

2.1. Sample processing

Pork loins from six different animals were collected from HKScan Ltd slaughterhouse (Forssa, Finland). The loins were excised at 6 h postmortem after standard slaughter and cooling procedures, vacuum

packaged and transported refrigerated to the meat laboratory at University of Helsinki. The loins arrived at 10 h postmortem and were stored overnight at 2 °C. Three batches of minced meat were prepared for this study. Each batch contained two isolated LTL muscles from different pork loins. At 24 h postmortem the LTL muscles were trimmed of visible connective tissue and external fat and minced in a grinder (LM-5P, Koneteollisuus Oy, Finland) through a plate with pore size of 3 mm. The minced meat was kept on ice until it was divided into two parts; one part was assigned to one or two freezing and thawing cycles, and another was exposed to different pH at high ionic strength conditions.

2.1.1 Freezing and thawing cycles

Minced meat patties (around 9 cm in diameter and 1.5 cm of thickness) made by a round burger mold were individually weighed (around 80 g) and vacuum packaged, and then subjected to one of five groups with different freezing-thawing cycles: 1) non-frozen control; 2) fast and 3) slow freezing followed by thawing (1 cycle); 4) repeated fast and 5) repeated slow freezing followed by thawing (2 cycles). The fresh control was directly analyzed after 24 h storage at 2 ± 1 °C in a cold room (Huurre, Vantaa, Finland). The frozen-thawed samples of Group 2 and 3 were thawed at 2 ± 1 °C for 24 h, representing one freezing-thawing cycle. Subsequently, the fast frozen-thawed or slow frozen-thawed samples were subjected to one additional fast or slow freezing, respectively, followed by thawing at 2 ± 1 °C for 24 h, representing repeated fast or repeated slow freezing (2 cycles), respectively. Following the method of our previous study (Zhang & Ertbjerg, 2019), fast and slow freezing were conducted in a -80 °C freezer (Ultra Low, SANYO, Japan) and in a -18 °C walk-in freezer (Huurre), respectively. After 24 h of storage at -18 °C, the slow freezing samples were transferred to the -80 °C freezer to minimize any effects of frozen storage time and temperature. The freezing time which the meat temperature changes from -1 °C (beginning of

freezing) to -7 °C (80% of water being frozen), suggested by Anõn & Calvelo (1980) to be a characteristic freezing time, was < 15 min for the fast freezing and > 23 min for the slow freezing meat as shown in our previous study (Zhang & Ertbjerg, 2019). Freezing and thawing cycles were completed within one week. Following thawing, meat samples (around 3 g) were homogenized in 30 mL of cold MES buffer (75 mM KCl, 20 mM MES, 2 mM $MgCl_2$, 2 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), pH 5.5) using an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany, 10 s at 13,500 rpm), and were then centrifuged at 2,400×g for 10 min at 4 °C. The supernatant was decanted and the resultant myofibril pellet was washed twice in the same buffer to remove soluble proteins. The ultimate pH of the LTL muscles at 24 h postmortem was 5.5 ± 0.1 measured by an insertion electrode (Mettler-Toleda Inlab 427), and freezing and thawing cycles did not affect pH of the minced meat in this study. Each group was analyzed for purge loss (including thaw loss for frozen-thawed samples), and the myofibrils isolated from each group were used for determination of particle size distribution, water-holding capacity, surface hydrophobicity, and SDS-PAGE analysis.

2.1.2 Exposure of minced meat to different pH at high ionic strength

Portions of fresh minced meat were weighed at 24 h postmortem, and then mixed with KCl to reach a final concentration of 2 M after addition of 0.1 M, 0.2 M or 0.3 M of lactic acid (volume around 10% of the weight of meat) to adjust pH from pH 5.5 of the fresh meat to pH 5.4, 5.3, and 5.2, respectively. The control was added the same volume of water. The KCl amount needed to achieve 2 M in the liquid phase of the minced meat was calculated according to the measured chloride content in the supernatant after centrifuging minced meat samples added around 2 M KCl. The chloride content was determined by a Corning 926 Chloride Analyzer (Corning Ltd, Halstead, GB). The 2 M KCl used here corresponds to a

high ionic strength of 2 after calculation based on ion concentrations and charges, and is about 10 times larger than the original ionic strength of 0.19 in fresh meat (Offer & Knight, 1988). Six groups were included in this experiment: 1) fresh control at pH 5.5, no KCl added, 2) freezing control at pH 5.5, no KCl added, and fresh meat exposed to 3) pH 5.5, 4) pH 5.4, 5) pH 5.3 and 6) pH 5.2 at high ionic strength (2 M KCl added and pH adjusted with lactic acid). The freezing control used fresh minced meat which was frozen in a -18 °C walk-in freezer (slow freezing) and stored at the same temperature for 5 days followed by thawing at 2 ± 1 °C overnight. The exposure of minced meat to different pH values at high ionic strength was performed at 0 ± 1 °C for 4 h and the pH value of minced meat was measured by a direct insertion probe electrode (Mettler-Toleda Inlab 427). Myofibrils were subsequently isolated from the six groups by homogenizing 3 g meat mince in 30 mL cold MES buffer following the method of 2.1.1, and pH 5.5 and low ionic strength of myofibrils were restored following three times washing with MES buffer (pH 5.5). The resultant myofibril pellet was used for the determination of water-holding capacity, surface hydrophobicity and SDS-PAGE analysis, to obtain information on the denaturation of myofibrillar proteins that occurred during exposure to high ionic strength at different pH values.

2.2. Purge loss

Purge loss was calculated according to the difference between the initial weight and the weight after freezing-thawing cycles (expressed as percentage) and thus included the thaw loss of frozen-thawed samples. Fresh minced meat was weighed at 24 h postmortem (initial weight). Following 24 h storage at 2 ± 1 °C, the weight of minced meat from non-frozen group was recorded after being blotted dry with

filter paper. Samples subjected to freezing-thawing cycles were weighed individually after thawing at 2 ± 1 °C for 24 h, and the bags were not opened until the freezing-thawing cycles were completed.

2.3. Particle size

The particle size distribution of myofibril pellets was measured by a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK). Around 2 g pellets was suspended in MES buffer (see Section 2.1.1), and every suspension was analyzed five times using distilled water as dispersant. The refractive index was set to 1.46 and the absorption coefficient to 0.01. D(v,0.1) describes that the size of the particle for which 10% sample is below the corresponding size. D(3,2) is the surface-weighted mean diameter, and D(4,3) is the volume-weighted mean diameter of particles.

2.4. Water-holding capacity of myofibrils

Water-holding capacity of myofibrils isolated from 2.1.1 and 2.1.2 (where pH 5.5 and low ionic strength of the myofibril preparations had been restored by washing in a MES buffer) was determined according to previous method described by (Zhang & Ertbjerg, 2018). Myofibrils (around 1 g) were homogenized in 9 mL of the MES buffer (see Section 2.1.1) at 9,500 rpm for 30 s, and the water-holding capacity of myofibrils was defined as the amount of water held by 1 g of myofibrillar protein.

2.5. Surface hydrophobicity

Surface hydrophobicity of isolated myofibril pellets (2.1.1 and 2.1.2) was determined according to the method described by (Zhang & Ertbjerg, 2019). The measurements were done at pH of 5.5 and low ionic strength following the washing procedure of the myofibrils in cold MES buffer (section 2.1.2). The

protein content of the myofibril pellets following re-suspension in MES buffer was measured by the DC Protein Assay Kit. Myofibrillar protein suspensions were subsequently adjusted to 2 mg/mL. Next, 1 mL suspension was mixed with 80 μL of bromophenol blue (BPB) (1 mg/mL) followed by incubation for 10 min at room temperature and centrifugation at 10,000 x g for 3 min. The surface hydrophobicity was then represented as the amount of BPB bound by 1 mg of myofibrillar proteins.

2.6. SDS-PAGE and protein identification

Myofibrillar protein suspensions prepared for measurement of surface hydrophobicity was used for SDS-PAGE analysis. Diluted protein suspension (32.5 μL, adjusted to around 2.3 mg/mL) was mixed with 12.5 μL NuPAGE™ LDS Sample Buffer (4 ×) and 5 μL NuPAGE™ Sample Reducing Agent (10 ×) (Invitrogen, Carlsbad, CA) and heated at 70 °C for 10 min. An aliquot containing 15 μg protein was loaded onto NuPAGE™ Novex 3–8% Tris-Acetate gels. The electrophoresis was performed at 150 V for approximately 1 hour using NuPAGE™ Tris-acetate running buffer. The gels were captured by a digital camera after staining and destaining. The band of interest in the gel was excised and then the protein was identified following the method of Vaarala et al. (2014) by mass spectrometry analysis.

2.7. Statistical analysis

Three independent batches were used in this study, and two pork LTL muscles were involved in each batch. Triplicates were done for purge loss, particle size distribution, water-holding capacity, and surface hydrophobicity, resulting in 9 replicates for each determination. A representative SDS-PAGE gel image from one batch was shown and the results were replicated. Statistical analysis was performed using general linear model in the IBM SPSS Statistics 25 software. Batch number was arranged as a random

factor, and treatment (non-frozen, fast and slow freezing, repeated fast and repeated slow freezing) was included as fixed factor in the experiment of freezing and thawing cycles, and different combinations of pH and ionic strength as fixed factor in the exposure study. The significant differences between group means at a level at $P < 0.05$ were evaluated by the Bonferroni test.

3. Results

3.1. Freezing and thawing cycles

3.1.1. Purge loss

Purge loss was defined as the water loss of fresh minced meat during chilled storage for 24 h, and of frozen-thawed minced meat after thawing-storage for 24 h. Samples from freezing and thawing cycles showed greater purge loss ($P < 0.05$) than the non-frozen group (Fig. 1A). One freezing-thawing cycle increased significantly purge loss from 4.7% (fresh) to 7.9% (fast freezing) and 10.2% (slow freezing), resulting in around 30% greater purge loss in slow compared to fast freezing. Two freezing-thawing cycles showed no further increase ($P > 0.05$) of purge loss compared to one freezing-thawing cycle.

3.1.2. Water-holding capacity and surface hydrophobicity of myofibrils

Water-holding of minced meat is to a large extent influenced by the properties of the myofibrils. Repeated freezing and thawing significantly affected the water-holding capacity of myofibrils extracted from minced meat (Fig. 1B), and the order of the values between groups was fresh > one freezing-thawing cycle > two freezing-thawing cycles. Among frozen-thawed groups, a significant effect of freezing rate was observed. Myofibrils isolated from fast or repeated fast freezing groups showed larger ($P < 0.05$) water-holding capacity compared to that of slow or repeated slow freezing samples, respectively. Surface

hydrophobicity can be taken as an indicator of myofibrillar protein denaturation. The surface hydrophobicity of myofibrils followed an opposite trend compared to water-holding and increased ($P < 0.05$) in response to increased freezing and thawing cycles (Fig. 1C); the largest increase by around 31% was clearly observed in the repeated slow freezing group compared to the fresh group.

3.1.3. Particle size

The particle size distribution of myofibrils was used here to assess the aggregation of myofibrillar proteins caused by repeated freezing and thawing cycles (Fig. 2). Slow freezing caused larger values ($P < 0.05$) of D(3,2), D(4,3) and D(v,0.1) compared to the fresh samples and additional slow freezing-thawing cycle was observed to significantly increase the values of D(3,2) and D(v,0.1), whereas no effect was observed caused by fast and repeated fast freezing; this indicates that the myofibrils formed larger particles upon slow freezing and thawing.

3.1.4. SDS-PAGE

A representative SDS-PAGE pattern of myofibrils isolated from minced meat following freezing and thawing cycles is shown in Fig. 3. A distinct protein band migrating at a position around 160 kDa appeared only in the frozen-thawed samples, and the intensity of this band increased with freezing and thawing cycles. The protein band was excised from the gel and identified by mass spectroscopy as a myosin fragment derived from myosin-4 (MYH4) which has a native molecular weight of 224 kDa.

3.2. Exposure of minced meat to high ionic strength at different pH values

In this experiment we simulated the exposure of minced meat to lower pH values at high ionic strength (corresponding to 2 M KCl) to mimic changes of myofibrillar proteins in frozen-thawed meat by observing water-holding capacity, surface hydrophobicity and SDS-PAGE of isolated myofibrils.

3.2.1. Water-holding capacity and surface hydrophobicity of myofibrils

Freezing-thawing significantly decreased the water-holding capacity of myofibrils by around 18% compared to that of the non-frozen control (Fig. 4A). Exposure of minced meat to high ionic strength at pH 5.5 significantly increased the water-holding capacity of myofibrils compared to the non-exposed control (from 9.2 g to 10 g H_2O/g protein). This difference reflects irreversible structural modifications in the myofibrillar protein network induced by high ionic strength during the exposure, since the 2 M KCl used for increasing the ionic strength of minced meat was washed out of the myofibrils before the water-holding measurement. As freezing had the opposite effect on water-holding, high ionic strength exposure alone did not reproduce the structural modifications that occurred in myofibrils during freezing. However, exposure to high ionic strength at lower pH values (pH 5.4, 5.3 and 5.2) all showed progressively decreased ($P < 0.05$) water-holding capacity in comparison with the non-frozen control (Fig. 4A).When minced meat was exposed to high ionic strength at pH 5.3 the water-holding capacity of the isolated and washed myofibrils was similar to that of myofibrils from meat that had been slowly frozen and then thawed.

Myofibrils from frozen-thawed meat showed greater ($P < 0.05$) surface hydrophobicity as compared to the non-frozen control. Similarly, a clear effect ($P < 0.05$) toward an increase of surface hydrophobicity

was observed in the fresh minced meat exposed to high ionic strength, with increasing surface hydrophobicity at lower pH (Fig. 4B).

3.2.2. SDS-PAGE

Myofibrils from frozen-thawed meat showed a clear band at a position of around 160 kDa (Fig. 5), migrating at the same position as the fragment identified to originate from myosin-4 (MYH4) in the experiment of freezing and thawing cycles (Fig. 3). The appearance of the same band was observed in the non-frozen minced meat following exposure to high ionic strength, and its intensity increased in response to exposure at lower pH. Furthermore, an increase in a 97 kDa band was observed only in the non-frozen minced meat after exposure to high ionic strength, and its intensity increased in response to exposure at lower pH. This 97 kDa band most likely correspond to the protein recognized as phosphorylase by a previous study (Liu, Ruusunen, Puolanne, & Ertbjerg, 2014).

4. Discussion

4.1. A model mimicking protein changes in freezing

Freezing below -20 °C induces crystallization of > 90% of the water present in the muscle structure and this could theoretically result in a > 10-fold increase in ionic strength and proton concentration in the remaining non-frozen water phase. However, the buffer capacity of especially the structural proteins would partly absorb the excess protons. Therefore, it is to be expected that there will be a resultant decreased pH combined with concentrated ionic strength in the remaining unfrozen phase, thereby inducing protein denaturation in freezing. We here propose a model (Fig. 6) to provide new insights into

the mechanism of protein denaturation in frozen-thawed meat based on simulated exposure of fresh meat to high ionic strength (corresponding to 2 M KCl) and lower pH.

Myosin molecules are negatively charged at pH 5.5 and low ionic strength in postmortem meat (Offer & Knight, 1988). Increases in ionic strength has conventionally been regarded as the main theory to explain freezing-induced protein conformational changes and resultant denaturation (Calvelo, 1981; Shenouda, 1980). Therefore, the exposure to high KCl concentration only in our model (Fig. 6B) hypothetically simulates the effect of high ionic strength (without pH change) on myosin in freezing. The myosin rod has positively charged amino acids that can bind chloride ions, and also negatively charged side chains that attract potassium ions. The inner center of the myosin shaft contains a hydrophobic core with a hollow structure, whereas the surface is more hydrophilic. As suggested by Puolanne & Halonen (2010) chloride ions could bind to the hydrophobic core of the myosin shaft. In addition, chloride ions as small, weakly-hydrated chaotropes are easily repelled by water and absorbed to the hydrophobic amino acid side chains i.e. leucine, valine and alanine on the outer surface of the myosin shaft. The myosin rod could thus be expected to predominantly bind more chloride ions, and the potassium ions will stay in the water phase. Therefore, the net effect of adding KCl (Fig. 6B) is suggested to be an increase of the negative net charge of the myosin filaments, inducing repulsion of myosin molecules (Offer & Knight, 1988) and an increase of the myofibril cross-sectional area (Knight & Parsons, 1988; Wilding, Hedges, & Lillford, 1986). An effect of selective binding of chloride ions to myosin filaments is simultaneously increasing the surface area because more hydrophobic groups are exposed to water (Puolanne & Halonen, 2010). This could allow more bromophenol blue to access the hydrophobic sites on proteins (Chelh, Gatellier, & Santé-Lhoutellier, 2006), and potentially explain the increased surface hydrophobicity of myofibrils

observed following the exposure to high ionic strength (Fig. 4B). The larger water-holding capacity of myofibrils exposed to high ionic strength (Fig. 4A) could thus be a consequence of the balance between swelling of the myofilaments and increased surface hydrophobicity of myofibrils. However, exposure to high ionic strength at a constant pH of 5.5 did not cause decreased water-holding of myofibrils as occurred in frozen-thawed meat. We suggest that the water-accessible surface areas of the structural proteins within the myofibrils are exposed to a progressively decreased pH and concentrated solutes during freezing, potentially resulting in protein denaturation (Zhang & Ertbjerg, 2019). Our model (Fig. 6C) is therefore mimicking protein changes in freezing by the exposure to a lower pH combined with high ionic strength. High ionic strength could make myosin filaments more negatively charged, whereas a decrease of pH towards the isoelectric point will decrease the negative net charge of myofilaments. We speculate that the combined effect on net charges of a lower pH and high ionic strength will potentially lead to reduced negative net charges in the myofilaments, resulting in a transverse shrinkage of the fibers. Furthermore, the decreased pH combined with high ionic strength could cause more severe protein unfolding as evidenced by increased surface hydrophobicity of myofibrils (Fig. 4B). The overall effect of reduced negative net charges and increased surface hydrophobicity offers an explanation of why water-holding capacity of myofibrils is lower (Fig. 4A) following the exposure to decreased pH combined with high ionic strength. In this scenario it should be considered that the conditions of high ionic strength and reduced pH during freezing are reversible as the non-frozen water gradually becomes diluted as the ice crystals melts during thawing. However, the temporary exposure induced irreversible changes in myofibril structure as evidenced by the reduced water-holding and increased surface hydrophobicity (Fig. 1 and 4), increased particle size (Fig. 2), and fragmentation of myosin (Fig. 3). Similarly, in the mimicking experiment the temporary exposure to high ionic strength at lower pH values resulted in

irreversible changes as the washed myofibrils showed reduced water-holding (Fig. 4a), increased surface hydrophobicity (Fig.4b), and myosin fragmentation (Fig. 5).

Protein denaturation induced by freezing and thawing has been recognized in earlier studies (Chan, Omana, & Betti, 2011; Egelandsdal et al., 2019). The majority of water in the muscle fiber is trapped within the myofibrils, and denaturation of myofibrillar proteins can be linked to a reduction in the amount of water that can be held by myofibrillar proteins, and thereby contributing to generation of thaw loss. The decreased water-holding capacities of myofibrils both in frozen-thawed pork chops of *longissimus* muscle (Zhang & Ertbjerg, 2018, 2019) and frozen-thawed minced pork (Fig. 2 and 4) thus suggest that a considerable irreversible denaturation of myofibrillar proteins occurs during freezing and thawing. Swelling of myofibrils increase the amount of trapped water within myofibrils as hypothesized by electrostatic forces (Hamm, 1986). The contribution of ionic strength and pH to water-holding within myofibrils is significant. It is well known that the addition of NaCl to meat systems causes swelling and a resultant higher water-holding (Hamm, 1986). In agreement, Knight & Parsons (1988) observed swelling of isolated myofibrils when treated with increased NaCl concentration from 0.1 to 3 M. However, in low pH and higher ionic strength combinations Puolanne and Peltonen (2013) observed decreased water-holding of meat. Also, Zhang & Ertbjerg (2019) found decreased water-holding capacity of myofibrils following exposure of isolated myofibrils to low pH and high KCl concentration. These observations strongly suggest that a lower pH in combination with high ionic strength rather than only high ionic strength is causing the reduction of the amount of water that is held within myofibrils isolated from frozen-thawed muscle. Lower pH in combination with high ionic strength will likely lead to more

severe protein denaturation and thus contribute to reducing the amount of water that are held within myofibrils.

Surface hydrophobicity has been estimated based on the interaction of bromophenol blue with hydrophobic groups on myofibrils (Chelh et al., 2006). Factors such as pH and ionic strength could lead to conformational changes in the protein structure, causing exposure of hydrophobic residues to the surface thus affecting surface hydrophobicity (Lin et al., 1998). An increase in surface hydrophobicity of myofibrillar proteins was observed after being exposed to a lower pH (Sharedeh et al., 2015; Shen, Zhao, & Sun, 2019). Also increased ionic strength has been reported by Lin et al. (1998) to cause an increase in surface hydrophobicity, which can be attributed it to myosin filament swelling and exposure of its inner hydrophobic groups. In the current study, the exposure to either high ionic strength or high ionic strength combined with a decreased pH both caused a concomitant increase of surface hydrophobicity. However, an increase of surface hydrophobicity in the exposure to high ionic strength at constant pH was paralleled with a greater water-holding capacity of myofibrils, indicating that surface hydrophobicity only reflects some aspects of the denaturation of myofibrils. In agreement, Liu, Arner, Puolanne, & Ertbjerg (2016) observed increased surface hydrophobicity and reduced water loss in the presence of denatured sarcoplasmic proteins in pale, soft and exudative (PSE) like conditions, and they suggested that denatured sarcoplasmic proteins precipitated onto the surface of myofilaments and myofibrils contributed to the surface hydrophobicity. It has been reported by Liu et al. (2014) and Zhu, Ruusunen, Gusella, Zhou, & Puolanne (2011) that pre-rigor incubation at around 40 °C to stimulate PSE-like condition could induce the translocation of phosphorylase from the sarcoplasmic to the myofibrillar fraction, indicating considerable denaturation of the enzyme phosphorylase. In the present study, minced

meat was directly exposed to pH-KCl combinations, and this might potentially increase the access of soluble sarcoplasmic proteins to myofilament surfaces compared to the conditions found in freezing where less than 10% of the water is available, and thereby causing that more denatured phosphorylase precipitated onto myofilaments observed in this study (Fig. 5). Precipitation of phosphorylase after the exposure to pH-KCl combinations may thus explain the increased surface hydrophobicity as compared to that observed in freezing.

Overall, our data suggests that water-holding capacity of myofibrils is better than surface hydrophobicity in illustrating myofibrillar protein denaturation and the resultant thaw loss found in freezing-thawing. The reduced water-holding capacity of myofibrils could thus only be reproduced by the exposure of minced meat to high salt combined with a decreased pH. Therefore, we ascribed the protein changes in myofibrils in the freezing-thawing process to a lower pH in combination with increasing ionic strength in the non-frozen water rather than progressively increasing ionic strength.

4.2. Effects of freezing and thawing cycles in relation to freezing rate

Decreased water-holding capacity of myofibrils and increased surface hydrophobicity were both observed with additional freezing-thawing cycles (Fig. 1), suggesting that more severe myofibrillar protein denaturation occurred, independent of freezing rate. As discussed above, exposure to lower pH in combination with high ionic strength in the unfrozen water in freezing is hypothesized (Fig. 6) to cause protein denaturation. The water-accessible surface area of myofibrillar proteins would then repeatedly be exposed to pH-ionic strength combinations following two freezing-thawing cycles, potentially leading

to more pronounced protein denaturation independent of the freezing rate. The loss of sarcoplasmic proteins in the thaw drip may also affect myofibrillar proteins with increased freezing-thawing cycles. Yoon, Lee, & Hufnagel (1991) found that removal of water-soluble sarcoplasmic proteins facilitated freeze-induced contraction of myofibrils, leading to textural hardening of fish mince. Also Wagner & Anon (1985) observed decreased denaturation temperature for myosin and actin in the muscles depleted of sarcoplasmic proteins and connective tissue. These observations suggest involvement of sarcoplasmic proteins in the protection of myofibrils against freezing-induced denaturation. In the present study, compared to 4.7% in purge loss as a baseline in the fresh patties after 24h storage, fast freezing samples caused an increase of 3.2% units whereas slow freezing samples caused a larger increase of 5.5% units after thawing. Cheng et al. (2019) reported a significant increase of thaw loss of beef muscle up to 4 freezing-thawing cycles. No further increase of purge loss, however, was observed with one additional freezing-thawing cycle in the present study. This may be related to the determination method of purge loss in this assay, as the vacuum bags were not opened until analysis, possibly resulting in more reabsorption of thaw drip following freezing-thawing. Generally, fast freezing resulted in superior myofibrillar protein attributes compared to slow freezing, independent of freezing-thawing cycles; this was shown both in larger pieces of frozen-thawed muscle (Zhang & Ertbjerg, 2019) and minced muscle in this study.

Liu, Xiong, & Rentfrow (2011) observed a loss of myosin heavy chain in freeze-thaw abused pork samples. Also Yamamoto, Samejima & Yasui (1977) reported that bands between 140 and 200 kDa occurred after freezing and thawing of hen muscle, and they attributed it to the degradation of myosin heavy chain caused by the release of lysosomal cathepsins into sarcoplasm. Myosin-4 (MYH4) is one

isoform of the myosin heavy chain, and Lopez-Pedrouso et al. (2018) observed a stronger response of myosin-4 to proteolysis in dry-cured ham. Zhang & Ertbjerg (2018) found an increase of free Ca^{2+} followed by the activation of calpain-2 in frozen-thawed meat, supporting increased proteolytic potential. The appearance of myosin-4 fragment (around 160 kDa) following freezing and thawing (Fig. 3) could thus potentially be related to the proteolytic degradation of myosin heavy chain. In addition, chloride ions could potentially rupture the inner hydrophobic core of myosin filaments and then expose the inner hydrophobic groups to water thus possibly promoting the dissolution of myosin filaments into the molecules. Larger particles of myofibrils were formed following slow freezing and thawing, potentially suggesting an unfolding and aggregation of myofibrillar proteins. However, one additional freezing-thawing cycle in this study had no significant influence on particle size. This lack of effect on particle size may be explained by the combined influence of the increased protein degradation due to the activation of calpain-2 (Zhang & Ertbjerg, 2018) and the increased aggregation of myofibrillar proteins as suggested by the increased surface hydrophobicity (Fig. 1C).

5. Conclusions

Additional freezing-thawing cycle, independent of freezing rate, caused a decrease in water-holding capacity of myofibrils and a parallel increase of surface hydrophobicity as well as the appearance of a fragment around 160 kDa originating from myosin-4 observed in SDS-PAGE, indicating more pronounced denaturation of myofibrillar proteins. Therefore, freeze-thaw abuse, independent of the freezing rate, should be minimized in relation to myofibrillar protein attributes for further processing. This paper also illustrated myofibrillar protein denaturation in freezing mimicked by the exposure to either high ionic strength only or high ionic strength combined with a lower pH. Freezing-thawing

decreased water-holding capacity of myofibrils, but the exposure of fresh minced meat to only high KCl concentration conversely increased the water-holding capacity of myofibrils, indicating that the high ionic strength might not be the causative factor in the changes of myofibrillar protein due to freezing-thawing. Exposure to decreased pH combined with high KCl concentration resulted in reduced water-holding capacity of myofibrils, increased surface hydrophobicity of myofibrils and also the appearance of the myosin-4 fragment band, showing myofibrillar protein denaturation induced by a comparable mechanism to that observed in freezing. We thus argue in a model that the mechanism of myofibrillar protein denaturation caused by the freezing-thawing is based on the involvement of a decreased pH combined with increased ionic strength rather than increased ionic strength only in the unfrozen water.

Acknowledgments

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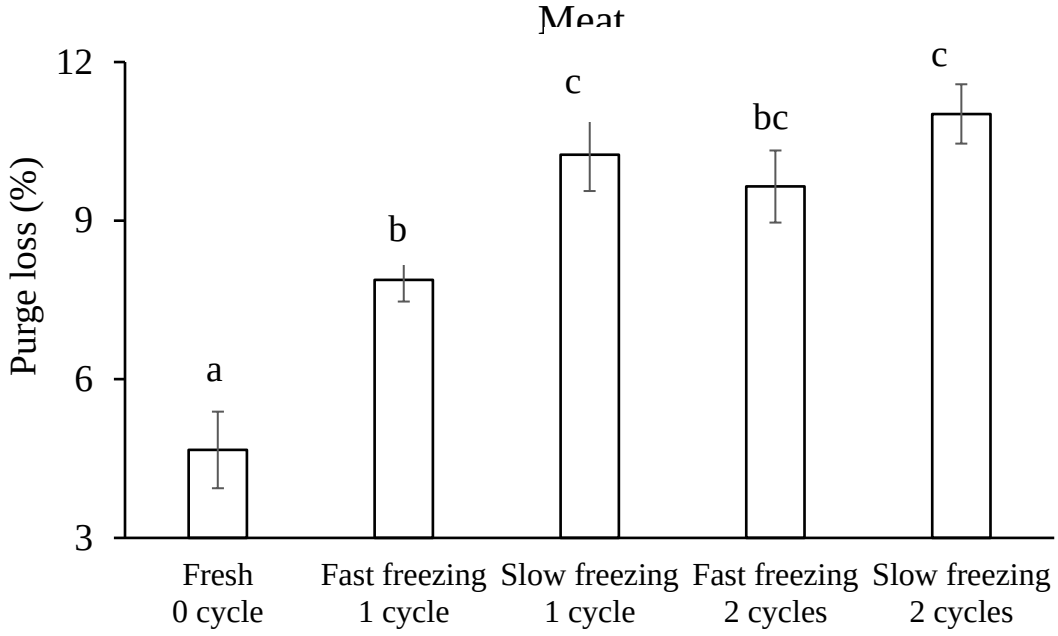
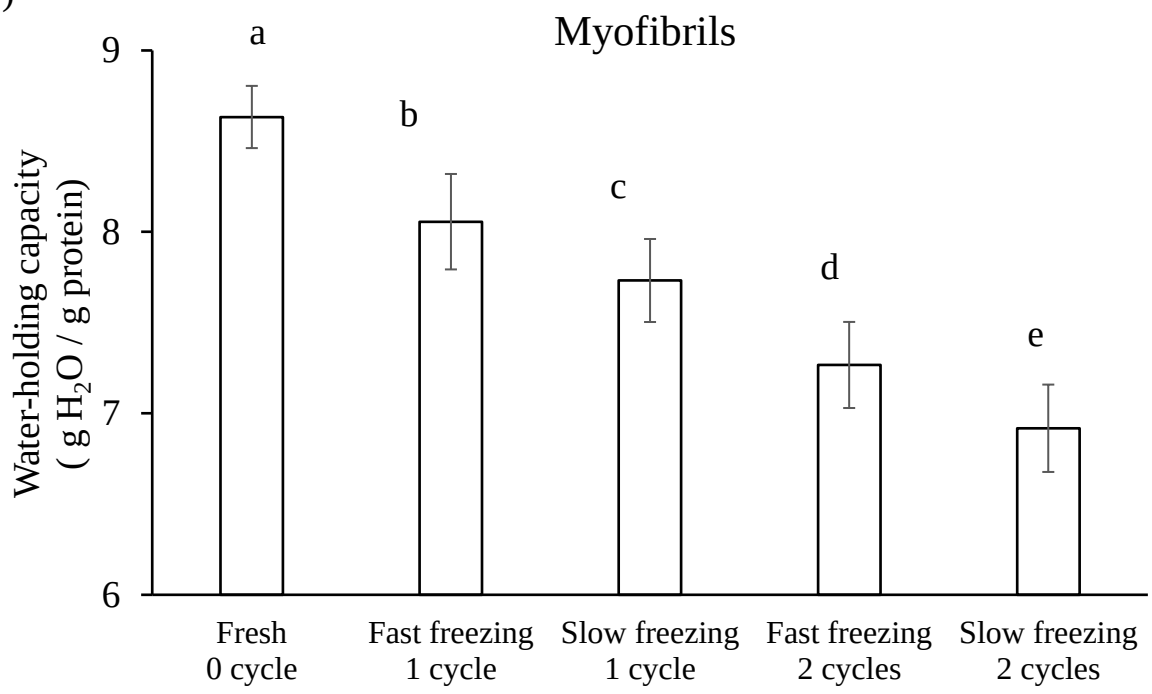
A)


Meat
Purge loss (%)
12
9
6
3
a
b
c
bc
c
Fresh
0 cycle
Fast freezing
1 cycle
Slow freezing
1 cycle
Fast freezing
2 cycles
Slow freezing
2 cycles

B)


Myofibrils
Water-holding capacity
(g H_2O / g protein)
9
8
7
6
a
b
c
d
e
Fresh
0 cycle
Fast freezing
1 cycle
Slow freezing
1 cycle
Fast freezing
2 cycles
Slow freezing
2 cycles

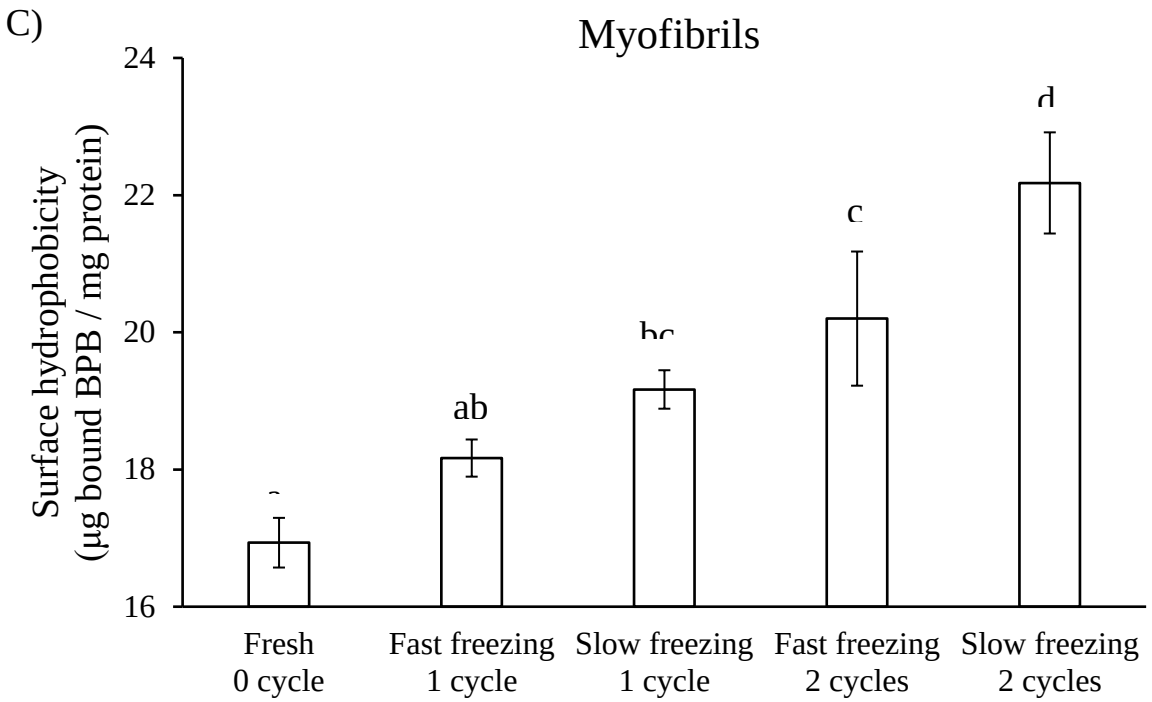
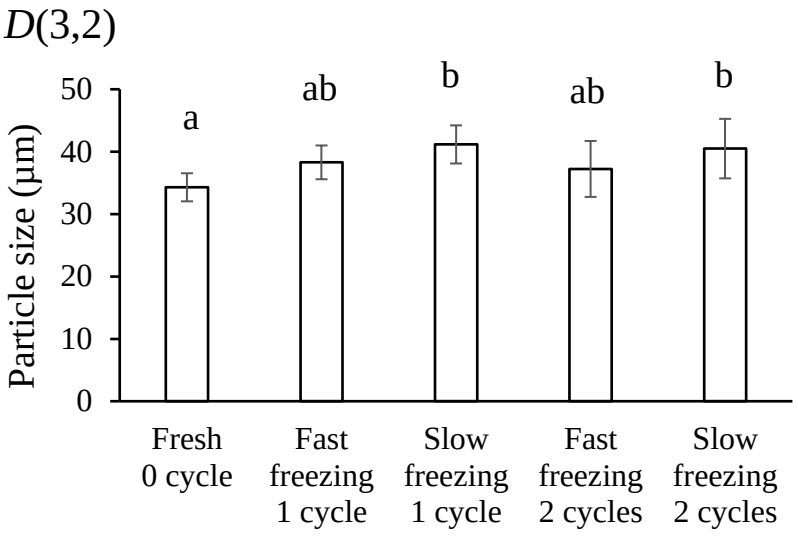
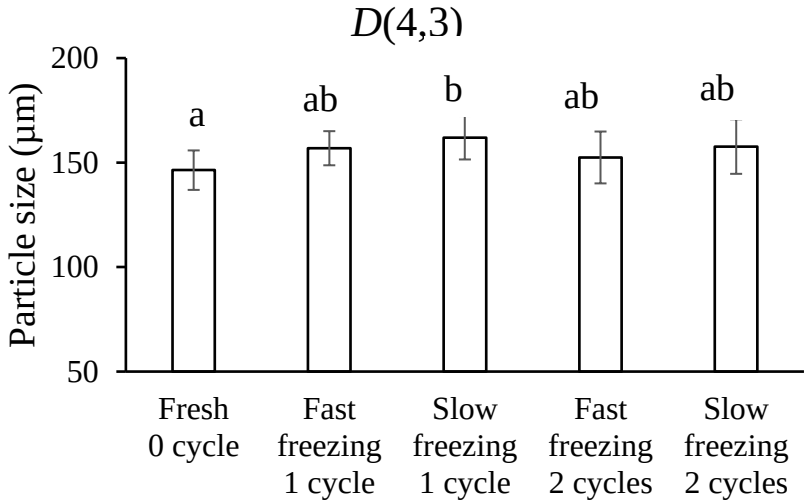


Fig. 1. Purge loss (%) of minced meat (A), water-holding capacity of myofibrils (B) and surface hydrophobicity of myofibrils (C) after 0, 1 and 2 freezing and thawing cycles. The fresh samples (0 cycle) were assigned to either fast or slow freezing, and were then thawed at 2 ± 1 °C for 24 h, representing one freezing-thawing cycle. Subsequently, the fast frozen-thawed or slow frozen-thawed samples were subjected to fast or slow freezing, respectively, followed by thawing at 2 ± 1 °C for 24 h, representing 2 cycles. Means ± standard errors are shown. BPB: bromophenol blue. a-e: Within each trait, mean values with the same letter do not differ ($P > 0.05$).

A)


D(3,2)
Particle size (µm)
50
40
30
20
10
0
a
ab
b
ab
b
Fresh 0 cycle
Fast freezing 1 cycle
Slow freezing 1 cycle
Fast freezing 2 cycles
Slow freezing 2 cycles

B)


D(4,3)
Particle size (µm)
200
150
100
50
a
ab
b
ab
ab
Fresh 0 cycle
Fast freezing 1 cycle
Slow freezing 1 cycle
Fast freezing 2 cycles
Slow freezing 2 cycles

C)

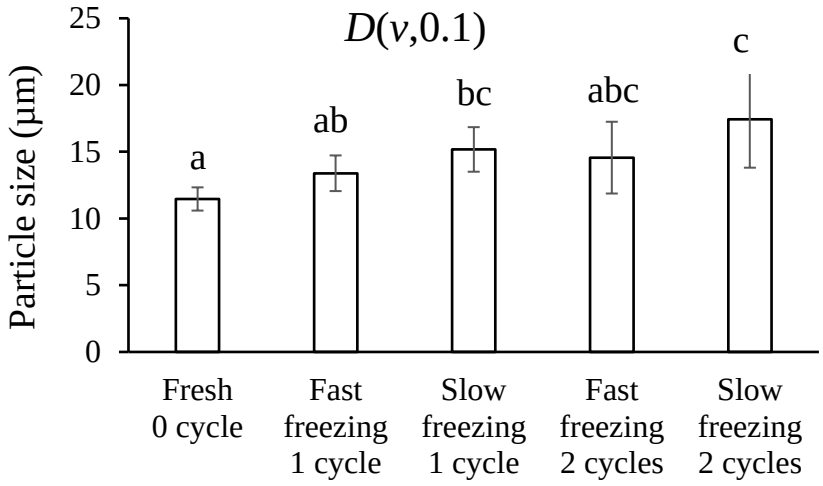

D(v,0.1)
Particle size (µm)
25
20
15
10
5
0
a
ab
bc
abc
c
Fresh 0 cycle
Fast freezing 1 cycle
Slow freezing 1 cycle
Fast freezing 2 cycles
Slow freezing 2 cycles

Fig. 2. Particle size as D(3,2) (A), D(4,3) (B) and D(v,0.1) (C) of myofibrils isolated from minced meat after 0, 1 and 2 freezing and thawing cycles. The fresh samples (0 cycle) were assigned to either fast frozen or slow freezing, and were then thawed at 2 ± 1 °C for 24 h, representing one freezing-thawing cycle. Subsequently, the fast frozen-thawed or slow frozen-thawed samples were subjected to fast or slow freezing, respectively, followed by thawing at 2 ± 1 °C for 24 h, representing 2 cycles. Means ± standard errors are shown. a-c: Within each trait, mean values with the same letter do not differ ($P > 0.05$).

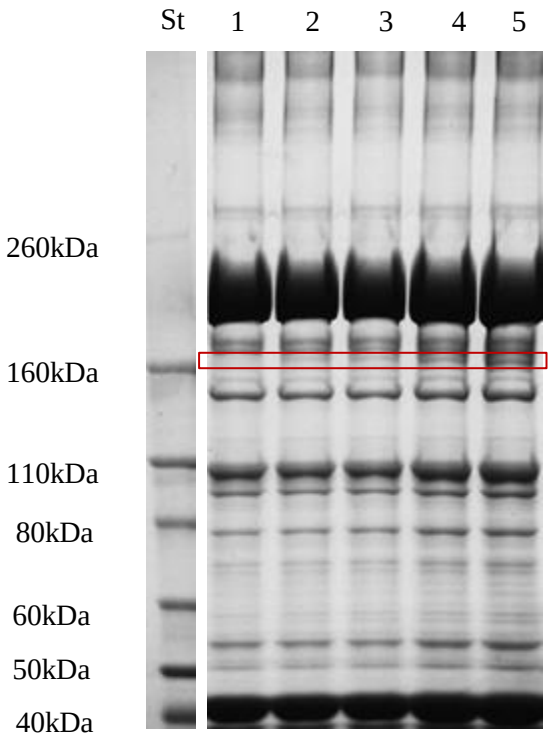
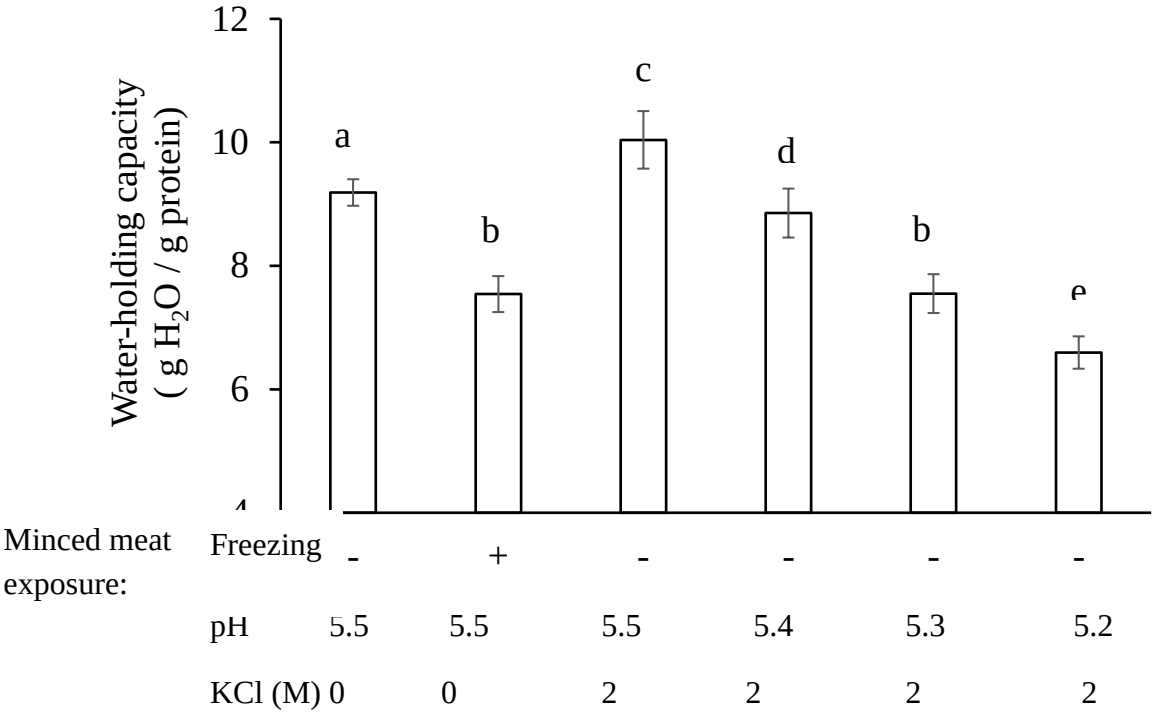


Fig. 3. Representative SDS-PAGE of myofibrils collected from minced pork after 0, 1 and 2 freezing and thawing cycles. St: molecular weight standard. Fresh, 0 cycle (1); Fast freezing, one freezing-thawing cycle (2); Slow freezing, one freezing-thawing cycle (3); Fast freezing, two freezing-thawing cycles (4); Slow freezing, two freezing-thawing cycles (5). The myosin heavy chain fragment around 160 kDa was identified as originating from myosin-4 (MYH 4) by mass spectrometry analysis.

Fig. 4A)


Water-holding capacity
(g H_2O / g protein)
12
10
8
6
4
a
b
c
d
b
e
Minced meat exposure:
Freezing - + - - - -
pH 5.5 5.5 5.5 5.4 5.3 5.2
KCl (M) 0 0 2 2 2 2

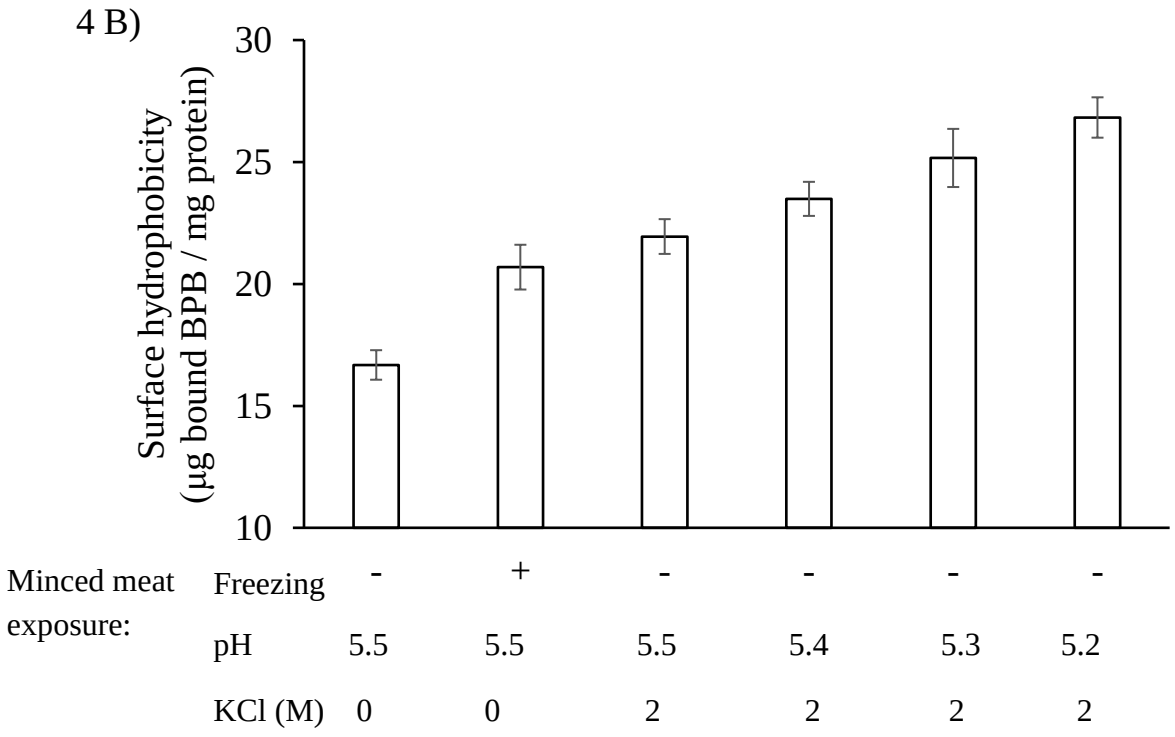


Fig. 4. Water-holding capacity (A), and surface hydrophobicity (B) of myofibrils. Minced meat was exposed to various pH-KCl combinations and thereafter myofibrils were washed in a MES buffer whereby the high salt was removed and pH was readjusted to 5.5 before the determination of water-holding capacity and surface hydrophobicity of the myofibrils. Treatments: Non-frozen control, pH 5.5 and no addition of KCl; Freezing followed by thawing overnight, pH 5.5 and no addition of KCl; Non-frozen, pH 5.5 and added 2 M KCl to minced meat; Non-frozen, pH 5.4 and added 2 M KCl to minced meat; Non-frozen, pH 5.3 and added 2 M KCl to minced meat; Non-frozen, pH 5.2 and added 2 M KCl to minced meat. Means ± standard errors are shown. BPB: bromophenol blue. a-e: Within each trait, mean values with the same letter do not differ ($P > 0.05$).

Fig. 5.

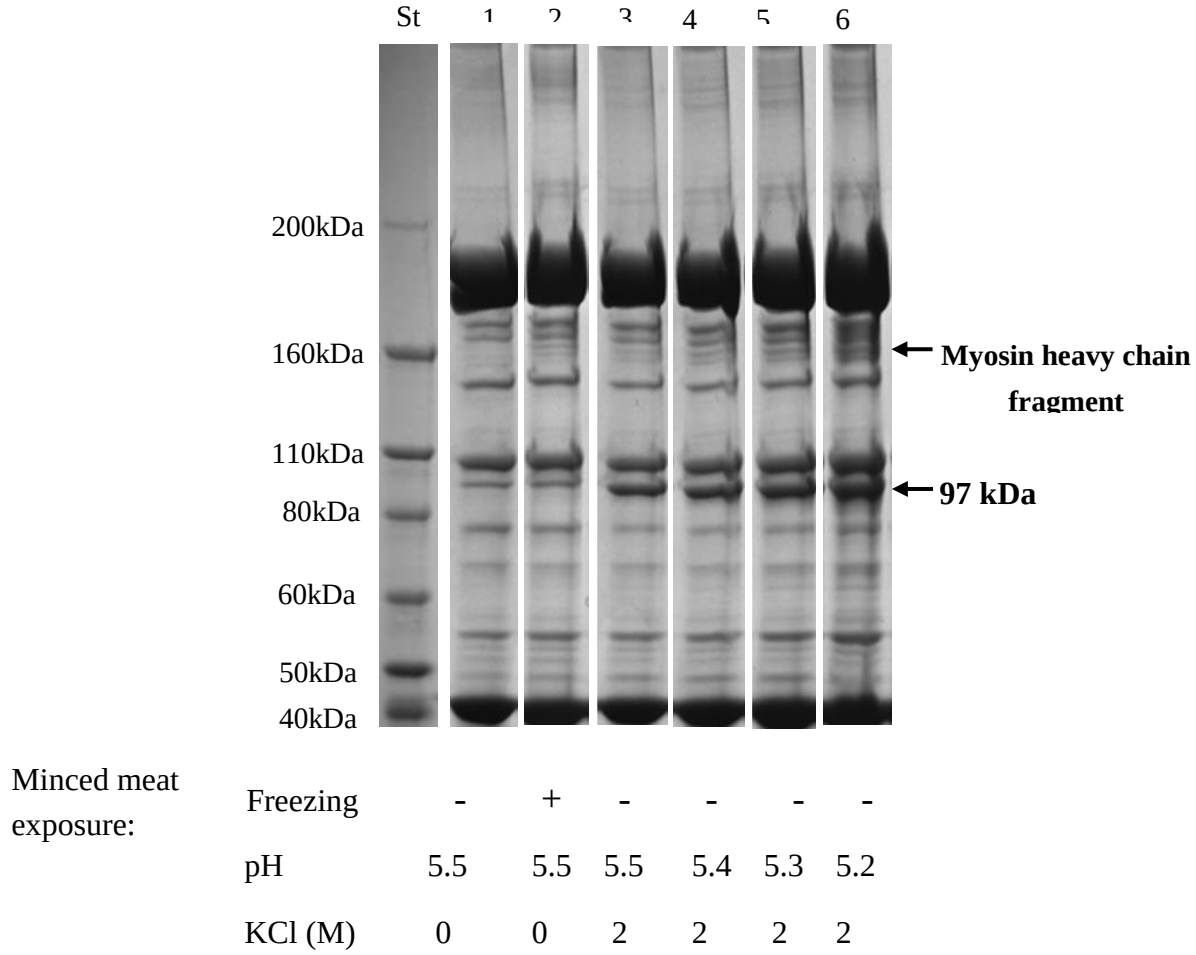


Fig. 5. Representative SDS-PAGE of myofibrils. Minced meat was exposed to various pH-KCl combinations and thereafter myofibrils were isolated and pH was readjusted to 5.5 in a MES buffer before the determination of SDS-PAGE. St is molecular weight standard. Treatment: Non-frozen control, pH 5.5 and no addition of KCl (1); Freezing followed by thawing overnight, pH 5.5 and no addition of KCl (2); Non-frozen, pH 5.5 and added 2 M KCl to minced meat (3); Non-frozen, pH 5.4 and added 2 M KCl to minced meat (4); Non-frozen, pH 5.3 and added 2 M KCl to minced meat (5); Non-frozen, pH 5.2 and added 2 M KCl to minced meat (6).

Fig. 6.

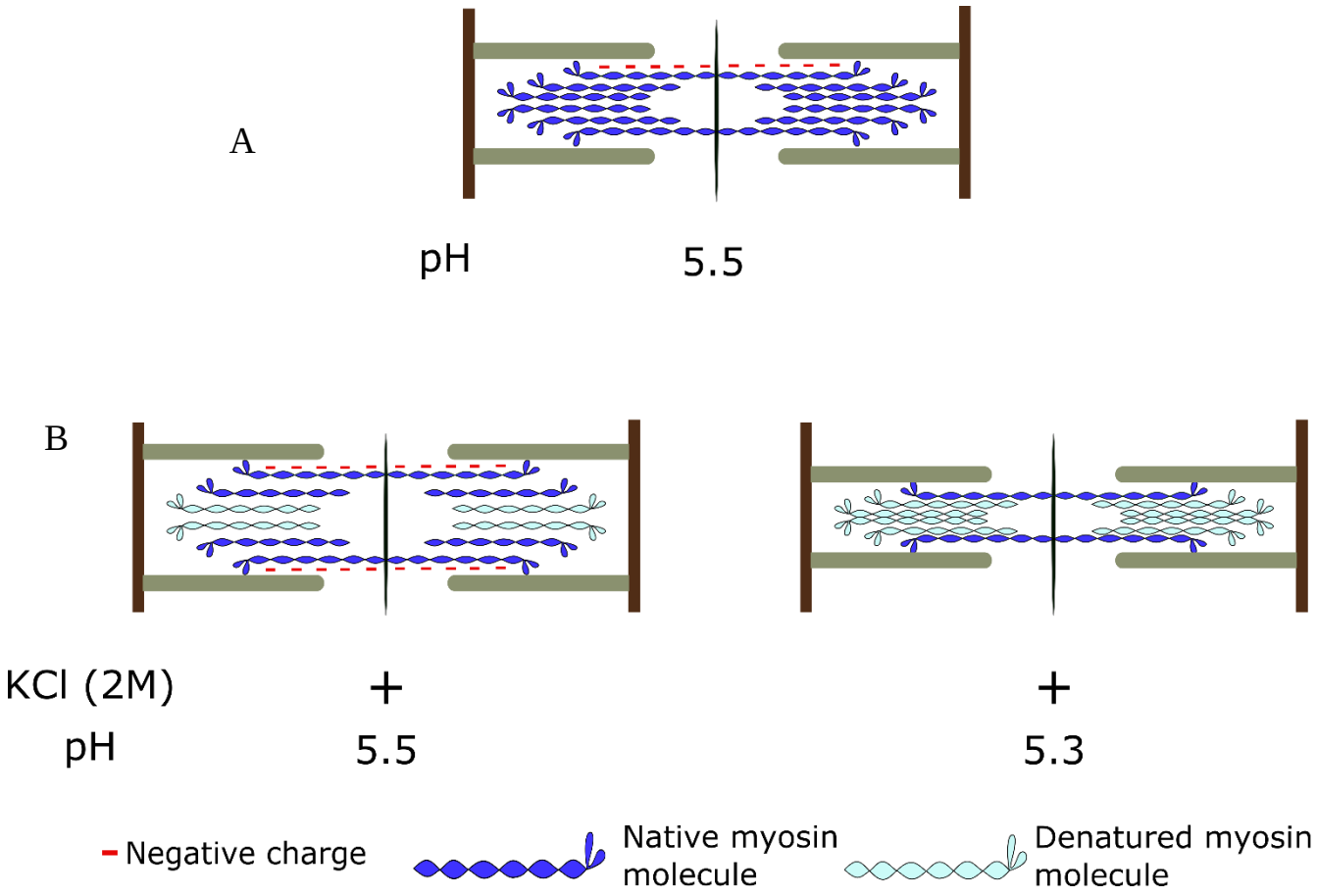


Fig. 6. Model illustrating possible events in freezing. The exposure to either high ionic strength (2 M KCl) or high ionic strength (2 M KCl) combined with a lower pH (5.3) and the effect on myosin filaments within the sarcomere is shown. (A) Native sarcomere in fresh meat. Myosin molecules are net negatively charged at pH 5.5 and low ionic strength. (B) Hypothetical effect of only high ionic strength in freezing. The exposure to 2 M KCl causes an increase in negative charge density of myosin filaments, resulting in repulsion of myosin molecules and transverse swelling of the sarcomere, thereby increasing water-holding. More hydrophobic groups are exposed on the outside, resulting in increased myosin filament surface hydrophobicity. (C) Hypothetical effect of high ionic strength combined with lower pH in freezing. The exposure to a pH-KCl combination causes a reduction of negative charges (no negative charges are shown). KCl increases negative charges, however, the decrease of pH also increases positive charges. Therefore, the combined effect results in reduced negative net charges of myosin filaments and transverse shrinkage of the sarcomere, leading to reduced water-holding. More myosin molecules are denatured by pH and KCl, leading to more pronounced increase of surface hydrophobicity. The exposure to pH-KCl combinations were mimicking the changes of myofibrillar proteins observed in freezing-thawing.