Department of Food and Nutrition
University of Helsinki
Finland

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Myofibrillar protein denaturation in freezing-thawing: Effect on thaw loss

Yuemei Zhang

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in Biocenter 2 hall 2041, Viikki, on 17th December 2020, at 13 o' clock.

Helsinki 2020

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ISBN 978-951-51-6881-8 (paperback)
ISBN 978-951-51-6882-5 (PDF)
ISSN 0355-1180

Unigrafia
Helsinki 2020

Abstract

The thesis aimed to study the effects and mechanisms of freezing-thawing treatments on myofibrillar protein denaturation and to explore the role of myofibrillar protein denaturation in causing the formation of thaw loss.

The freezing-thawing of porcine *M. longissimus thoracis et lumborum* (LTL) increased the free Ca^{2+} concentration and the subsequent chilled storage promoted an accelerated decrease of activities of calpain-1 and calpain-2, compared to unfrozen meat. Proteasome activity was observed to be around 40% lower after freezing-thawing. The observed increased purge loss and decreased water-holding capacity (WHC) of myofibrils indicated myofibrillar protein denaturation occurring during the freezing-thawing treatment.

In the investigation of freezing-induced denaturation of myofibrillar and sarcoplasmic proteins of LTL in relation to freezing rate, fast frozen samples (cold metal plate/-80°C) had a characteristic freezing time of 12 min, while samples frozen at slow rate (air/-20°C) had a freezing time of around 174 min. Slow freezing led to around 30% larger thaw loss in parallel with reduced WHC and increased surface hydrophobicity of myofibrils, indicating more severe myofibrillar protein denaturation in slow compared to fast freezing. A model is proposed to explain the importance of myofibrillar protein denaturation in relation to the freezing rate in the origin of thaw loss: In slow freezing, protons are accumulated with concentrating solutes in the unfrozen water leading to a decline of pH causing denaturation of structural proteins. In fast freezing small ice crystals might trap protons inducing less decline of pH and thus less myofibrillar protein denaturation and reduced thaw loss when compared with slow freezing. Sarcoplasmic protein denaturation also was shown to occur in freezing-thawing as evaluated by differential scanning calorimetry and tryptophan fluorescence properties of drip, which was, however, independent of freezing rate.

The role of decreased pH (from pH 5.5 to 5.2), combined with high ionic strength (2 M KCl), in causing myofibrillar protein denaturation was studied by exposing fresh minced meat to either high ionic strength only or to high ionic strength with decreased pH to mimic conditions estimated to be in the unfrozen water of frozen meat during freezing. Exposure to high ionic strength caused an increase of WHC of the isolated myofibrils, whereas exposure to high ionic strength combined with low pH reduced WHC and increased surface hydrophobicity of the myofibrils. These results suggest that decreased pH combined with increased ionic strength in the unfrozen water of frozen meat largely

would explain myofibrillar protein denaturation and thus the thaw loss occurring in frozen-thawed meat.

The storage at -3 °C of fast or slow frozen pork prior to final thawing at 2 °C diminished the impact of the freezing rate on myofibrillar protein attributes, and differences between fast and slow freezing were no longer significant for WHC and surface hydrophobicity of the isolated myofibrils when frozen samples subsequently were kept at -3 °C for 7 days. The results suggest that a marked myofibrillar protein denaturation is taking place with extended storage time at -3 °C.

In conclusion, freezing-thawing increases water loss in meat and slow freezing causes a higher increase when compared to freezing at fast rate. Myofibrillar and sarcoplasmic protein denaturation occurs in freezing and thawing. The rate of freezing produces a significant effect in the myofibrillar fraction: A slow freezing will develop a more severe protein denaturation than a fast freezing. Consequently, the myofibrillar protein denaturation, related to the freezing rate, is proposed to contribute to the generation of thaw loss. However, a subsequent storage of frozen meat at -3 °C before final thawing seems to diminish the beneficial effects of fast freezing on myofibrillar protein characteristics, compared to slow freezing, due to an additional protein denaturation. Therefore, it is recommended that meat industry adjusts the thawing capacity to minimize the passage time in temperature of -3 °C in meat.

Acknowledgements

The work in the current thesis was carried out in Meat Technology group, Department of Food and Nutrition, Faculty of Agriculture and Forestry, University of Helsinki during the past four years from 2016-2020. Financial resource is from the scholarship provided by the China Scholarship Council. The international conferences that I have attended were supported by the Chancellor’s Travel Grant / Doctoral Programme in Food Chain and Health.

I would like to express the depth of my gratitude to my supervisor, Associate Professor Per Ertbjerg for giving me a lot of support during my entire doctoral study. Thank you for always being nice and patient with me. I truly appreciate your attitude to the scientific work that you always pay attention to every detail even for reading scientific papers. I am grateful for all the discussions that I had with you which was quite informative and inspired for operating my research experiments. I would like to give my special gratitude to my best Finnish friend, Professor Emeritus Eero Puolanne. It is always enjoyable to have a conversation with you. You are always being supportive of me not only in meat technology but also in life. Special thanks to you and your wife Eila for inviting me to your cottage which gave me opportunities to experience the wonderful Finnish summer and winter.

I greatly acknowledge Professor Robyn D. Warner and Associate Professor Brad Kim for their valuable comments and suggestions during the pre-examination of this thesis. I also want to thank my follow-up group members Professor Frederick Stoddard and Associate Professor Jiao Liu for their useful suggestions to improve my doctoral research.

I give my warm gratitude to my dear colleagues Yulong Bao, Zhen Zeng, Jian Lyu, Luigi Pomponio, Arianna Magro, and Binbin Li. It is my pleasure to share office and lab with you. I am truly grateful for the technical support from Mikko Kangas, Taru Rautavesi, Jutta Varis, Outi Brinck, and other technicians in the lab. My dear friends Yaqin Wang, Xin Huang, Chong Xie, Hongbo Zhao, Fengyuan Liu, and Xiaocui Han, thank you for all the wonderful time we spent together in the university and after work.

Finally, my warmest thanks are given to my family in Chinese:
感谢爸爸妈妈无私的爱与理解，对于我的每次选择，你们总是一如既往的支持。感谢爷爷奶奶对我的关心记挂和平时的嘘寒问暖。感谢弟弟一直默默地支持。感谢所有关心爱护我的家人，是因为一直有你们作为我的后盾，我才能无所顾忌的追逐梦想，从不畏惧向前！

Helsinki, December 2020

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Paper I

Zhang, Y., & Ertbjerg, P. (2018). Effects of frozen-then-chilled storage on proteolytic enzyme activity and WHC of pork loin. *Meat Science, 145*, 375-382.

Paper II

Zhang, Y., & Ertbjerg, P. (2019). On the origin of thaw loss: Relationship between freezing rate and protein denaturation. *Food Chemistry, 299*, 125104.

Paper III

Zhang, Y., Puolanne, E. & Ertbjerg, P. (2020). Mimicking myofibrillar protein denaturation in frozen-thawed meat: Effect of pH at high ionic strength. *Food Chemistry, 338*, 128017.

Paper IV

Zhang, Y., Magro, A., Puolanne, E., Dalle Zotte, A., & Ertbjerg, P. (2020). Myofibrillar protein characteristics of fast or slow deep frozen pork during subsequent storage at -3 °C. Manuscript.

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III: The study was planned by Yuemei Zhang and Per Ertbjerg together. The experimental analyses and manuscript preparation were done by Yuemei Zhang. Per Ertbjerg and Eero Puolanne gave comments and suggestions during the manuscript preparation.

IV: The study was planned by all the authors. The experimental analyses were done by Yuemei Zhang and Arianna Magro, and the manuscript preparation was done by Yuemei Zhang. Per Ertbjerg, Eero Puolanne and Antonella Dalle Zotte gave comments and suggestions during the manuscript preparation.

Abbreviations

ANS	1, 8-anilino-naphtalene-sulfonate
ATP	adenosine triphosphate
BF	*M. biceps femoris*
BPB	bromophenol blue
CPA	cis-parinaric acid
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene-bis(oxyethylenenitrilo)tetraacetic acid
HSD	honest significant difference
p*I*	isoelectric point
LTL	*M. longissimus thoracis et lumborum*
MES	2-(N-Morpholino) ethanesulfonic acid hydrate
NMR	nuclear magnetic resonance
PM	*M. pectoralis major*
PSE	pale, soft and exudative
SD	*M. semitendinosus*
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	*M. semimembranous*
Tris	tris(Hydroxymethyl)aminomethane
Z-GGL-AMC	carbobenzoxy-Gly-Gly-Leu-7p-amido-4-methylcoumarin

1. Introduction

Freezing has traditionally been recognized as one of the most effective methods to prevent bacteria-induced deterioration and to preserve meat and meat products. Water accounts for around 75% of fresh lean meat, and the majority of water in the muscle is located within the myofibrils (Huff-Lonergan & Lonergan, 2005). However, the process of freezing and frozen storage generally alters the water content and distribution within the meat tissue, causing a substantial increase of water loss and poorer WHC after thawing (Leygonie, Britz, & Hoffman, 2012a). More importantly, this will result in significant losses of meat weight and valuable nutrients in the exudate, and consequently decrease economical and nutritional values as compared to fresh meat. Aroeira et al. (2016) found that the amount of drip was four times higher in frozen-thawed beef compared to that of fresh. Ice crystallization as myowater is progressively frozen out results in an increase of solute concentration in the unfrozen water within the muscle structure, thus potentially causing quality defects of thawed meat, often shown as protein denaturation and oxidation, discoloration, and texture issues (Ballin & Lametsch, 2008; Kim, Kim, Seo, Setyabrata, & Kim, 2018; Leygonie et al., 2012a). In addition, the wet surface of frozen-thawed meat resulting from the released thaw exudate decrease the willingness of consumers to purchase the products. More importantly, when frozen-thawed meat is used for preparation of meat products, inferior water-holding capacity and protein properties may negatively affect the juiciness, tenderness and natural yield after processing, which consequently remains a significant problem for either the industry or consumers.

It is currently accepted that mechanical damage to muscle fibers induced by ice crystal formation is the main driving force for the generation of thaw loss. The size and location of ice crystals formed inside and outside of muscle fibers is affected by the freezing rate and is believed to be a decisive factor for thaw loss formation (Hamm, 1986; Leygonie et al., 2012a; Li, Zhu, & Sun, 2018). The characteristic freezing time (t_c) has been defined as the time for the meat temperature to decline from -1 °C to -7 °C, an interval where 80% of water is frozen out (Bevilacqua, Zaritzky, & Calvelo, 1979). Fast freezing, recognized as $t_c < 15$ min, forms numerous small intra- and extra-cellular ice crystals, whereas slow freezing at $t_c > 23$ min often produces large ice crystals located unevenly in the extracellular area thus leading to more cryo-damages to muscle fibers and consequently higher amount of thaw loss as compared to fast freezing (Grujić, Petrović, Pikula, & Amidžić, 1993; Kim et al., 2018).

Protein is the second largest component within meat tissue. Myofibrillar proteins, accounting for 60% to 70% of the total meat protein, play an important role in maintaining the water within the muscle structure, and any protein denaturation that takes place in freezing and thawing might potentially be linked to inferior WHC and consequently the generation of thaw loss. Hence, it would be reasonable to investigate the importance of protein denaturation in the generation of thaw loss.

Hamm (1961) proposed in the previous century the idea that freezing and prolonged frozen storage could induce denaturation of meat proteins. Several recent studies have reported freezing-induced protein denaturation in terms of decreased protein solubility, Ca^{2+}-ATPase activity, and increased surface hydrophobicity (Chan, Omana, & Betti, 2011; Qi et al., 2012). However, a role of freezing-induced protein denaturation for thaw loss formation has over the years been refuted by some studies since they did not find any significant influence of the freezing rate on protein denaturation considering protein solubility and surface hydrophobicity (Farouk, Wieliczko, & Merts, 2003), or denaturation enthalpy (Ngapo, Babare, Reynolds, & Mawson, 1999). The relative role of protein denaturation in the formation of thaw loss is thus still not well understood.

The contribution of high ionic strength to protein denaturation is well known to be significant, and Lin & Park (1998) observed decreased myosin solubility and increased surface hydrophobicity when salmon muscle was exposed to ionic strength above 1.5. Nevertheless, several studies have documented a positive role of salt on WHC of meat tissue, showing increased WHC with the addition of sodium chloride to a meat system corresponding to a high ionic strength (Hamm, 1986; Jiang, Nakazawa, Hu, Osako, & Okazaki, 2019a; Puolanne & Peltonen, 2013). Protein denaturation has traditionally been attributed to concentrating solutes in the unfrozen water, mechanical disruption caused by ice crystals, and dehydration of muscle fibers (Xiong, 1997). However, considering that interactions of myofibrillar protein and water strongly influence WHC (Hamm, 1986; Puolanne & Halonen, 2010), it can be questioned if increased ionic strength in the unfrozen water could be directly responsible for protein denaturation that occurs during freezing in relation to the loss of WHC within the meat tissue.

The combination of chilled and frozen storage has been reported to produce frozen meat with better WHC and tenderness compared to only frozen-thawed meat as reviewed by Coombs, Holman, Friend, & Hopkins (2017). Frozen-then-chilled storage could potentially offer some flexibility in the production process for the industry. Some studies have reported increased myofibril fragmentation

index, and accelerated desmin degradation in frozen-thawed beef muscle with subsequent chilled storage (Aroeira et al., 2016; Grayson, King, Shackelford, Koohmaraie, & Wheeler, 2014), suggesting greater proteolysis that occurred in freezing and thawing. More knowledge on the changes of proteolytic enzymes related to proteolysis and WHC during frozen-then-chilled storage is still needed.

In the present thesis, frozen-then-chilled storage and differences in the freezing rate were applied to study the effect of freezing on proteolytic enzymes and protein denaturation. The characteristic freezing time observed in the thesis were around 12 min for fast freezing and around 174 min for slow freezing of meat. By investigations of the exposure of fresh minced meat and myofibrils to lower pH levels at high ionic strength, new information on protein denaturation in freezing-thawing was provided. The ultimate aim was to expand the understanding on the involvement of protein denaturation in the generation of thaw loss.

The thesis first presents the literature review (Chapter 2): introducing 1) relevant knowledge on ice crystal formation and water loss in meat tissue during the freezing and thawing process; 2) protein denaturation that occurs during freezing and thawing and the mechanisms behind denaturation; 3) protein denaturation in relation to WHC of meat concerning roles of pH and ionic strength; and 4) hypotheses for causes of thaw loss. The objectives of the current thesis are shown in Chapter 3. Materials and methods used in the thesis are briefly presented in Chapter 4. A summary of results is described in Chapter 5 followed by a general discussion given in Chapter 6. Finally, conclusions and future perspectives are presented in Chapter 7 and 8, respectively.

2. Literature review

2.1. Ice crystal formation and water loss in meat tissue in freezing-thawing

2.1.1. Water distribution within the meat structure

Water is the most abundant component in meat tissue and constitutes around 75% in lean meat at time of slaughter (Offer & Knight, 1988a). Myofibrils occupy the largest part in the muscle fiber volume, for example, being up to 85% in guinea pig (Eisenberg & Kuda, 1975). The majority of the water is trapped within the myofibrils, and the others are often found in the space outside of myofibrils, i.e. between the myofibrils, between the muscle fibers and between the muscle fiber bundles (Offer & Cousins, 1992). In frozen meat, however, the water distribution is strongly related to the size and location of ice crystals formed during freezing (Li et al., 2018). It is thus important to understand water compartments distributed within the meat structure in order to provide information on the water in different compartments available for ice crystallization during freezing.

Three water compartments, recognized as bound water, entrapped water and free water, are generally involved in the muscle system in terms of the degree of immobilization (Bertram et al., 2001; Pearce, Rosenvold, Andersen, & Hopkins, 2011). Low-field NMR proton relaxometry has been used intensively as a technique for studying WHC within the meat matrix (Zhu, O'Farrell, Bouquet, Lunde, Egelandsdal, Alvseike, et al, 2016; Zhu, O'Farrell, Hansen, Andersen, Berg, & Egelandsdal, 2017). Three water fractions have by Bertram et al. (2001) been reported to account for 1-4%, 85-96%, and 2-10% of the total water amount, respectively, as measured by the relaxation times in low-field NMR relaxometry. Water as a dipolar molecule can bind to the charged groups at the surface of macromolecules, such as proteins. This water population in the muscle structure is thus defined as the bound water which usually exists in the vicinity of proteins. Bound water is highly resistant to freezing, for example, it is not easily freezable even at subzero temperatures below -40 °C (Aktas, Tulek, & Gokalp, 1997; Pham, 1987). Approximately 0.5 g of water is estimated to be tightly bound to 1 g of protein, and this may result in about 8 % of the total water amount being recognized as bound water, considering that the total protein concentration within the muscle tissue is around 200 mg/g (Huff-Lonergan & Lonergan, 2005). Bound water accounts for a relatively small population of the total water, and is not considered to contribute to drip formation during freezing and thawing (Offer & Knight, 1988b).

The other water populations within the muscle structure, often referred to as the entrapped and the free water, are easily converted to ice crystals during freezing. Xanthakis, Havet, Chevallier, Abadie, & Le-Bail (2013) reported the freezable water amount to be about 88% in frozen pork. The entrapped (or immobilized; Hamm, 1986) water is often firmly held within the myofibrils, mainly dependent on a balance between the electrostatic forces between the charged filaments and the osmotic pressure, as well as the structural constraint forces e.g. exerted by Z-disks and cross-bridges (Offer & Knight, 1988a; Puolanne & Halonen, 2010). Thus, the entrapped water is directly linked to the WHC of meat, thereby affecting meat quality traits, e.g. drip loss, juiciness, and firmness. This water population would not flow freely from early postmortem meat tissue, yet it is vulnerable to temperature and pressure. The mechanical disruption of muscle fibers and structural proteins denaturation occurring in freezing result in the migration of the entrapped water to reach the extracellular space where part of it possibly becomes free water and can be lost as exudate upon thawing (Huff-Lonergan & Lonergan, 2005). Free water exists between the myofibrils or in the extracellular spaces, i.e. between the muscle fibers and between the muscle fiber bundles. These water molecules are mainly restricted by the capillary force within the muscle structure (Offer & Knight, 1988a), and therefore are easily to be lost by gravity. Three water populations within different compartments and their relevant properties in relation to freezing are summarized below in Table 1.

Table 1. Summary of water populations and relevant properties in meat.

	Bound water	**Entrapped water**	**Free water**
Amount	Up to 8%	About 85%	Up to 10%
Compartment[1]	Bound to protein side chains	Within the myofibrils	Between the myofibrils Extracellular spaces
Main Forces[2]	Hydrogen bond	Electrostatic forces Osmotic pressure Structural constraint forces	Capillary force Structural constraint forces
Resistant to freezing	Stronger	Weaker	Weaker
Contributing to thaw loss	Nearly none	Part of fraction	Largest fraction
Factors affecting mobilization[2]	Not easily mobilized	Rigor process Protein denaturation	Gravity

Superscript 1-3: references: 1. Pearce et al., 2011; Offer & Cousins, 1992; 2. Offer & Knight, 1988a; Huff-Lonergan & Lonergan, 2005.

2.1.2. Ice crystal formation

Freezing of meat usually consists of three distinct stages (Fennema, Powrie, & Marth, 1973): a cooling process to decline the temperature of meat to reach the initial freezing point (below -1 °C), a phase changing stage where most of the muscle water starts to be frozen out, and a final stage to continue cool until reaching the final desired temperature for frozen storage. As shown in Fig. 1, the temperature interval from below -1 °C to -7 °C belongs to the second stage and is defined by Anõn & Calvelo (1980) as the critical temperature zone where 80% of freezable water is frozen accompanied with concentrating solutes in the unfrozen water (Finn, 1932). The product usually enters into this critical zone at the early beginning of freezing and then remains for a relatively long duration at which quality deterioration occurs, probably being attributable to the exposure of meat protein to the concentrating aqueous solution (Xiong, 1997). The time of the product to pass through this critical temperature zone is thus crucial to the freeze damage.

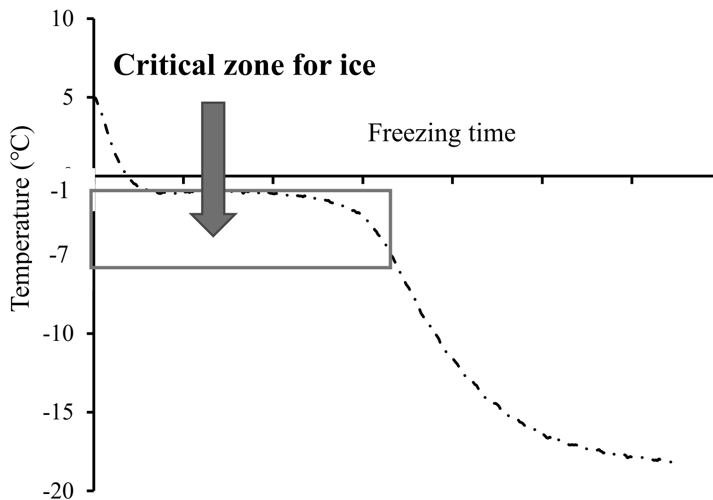

Fig. 1. An illustration of the freezing curve showing the critical temperature zone for ice crystallization.

The formation of ice crystals in the second stage is divided into two successive phases: nucleation, followed by the growth of ice crystals (Calvelo, 1981). Ice nucleation is an activated process. Supercooling, defined as the difference between the practical temperature and that of the solid-liquid equilibrium, is regarded as the main driving force of nucleation. The larger supercooling thus permits a greater number of nuclei per unit volume to generate. Once the nuclei have been generated, the following phase is defined as growth of ice crystals at which water molecules gradually accumulate at the surface of the formed nuclei as reviewed by Zhu, Zhou, & Sun (2019).

The initial freezing point between intra- and extracellular spaces is different within the meat tissue. Hamm (1986) concluded that the extracellular ice formation begins at around -1.2 °C, but a lower freezing temperature of around -1.6 °C is required for intracellular ice formation. The existence of intracellular ice is highly related to the magnitude of the supercooling occurring in freezing. However, the supercooling reached in the intracellular space is lower than that in the extracellular space (Bevilacqua, et al., 1979). This effect is likely attributable to the existence of a higher solute concentration inside of the muscle fiber compared to outside. The nucleation during freezing of muscle usually begins in the extracellular area. However, an intracellular ice nucleation could also be expected to occur at the beginning of freezing when the temperature within the muscle fiber declines sufficiently for the formation of intracellular nucleation. The thermal conductivity of ice is four times greater than

that of water, and therefore, the heat could dissipate out of the muscle via extracellular ice crystals allowing the freezing to continue there. The intracellular freezing will then start later. The situation is, however, inverse during thawing since there will be insolating water layer in the surfaced layers resisting the heat penetration.

It is generally accepted that the freezing rate significantly influences the size and location of ice crystals formed inside and outside of the muscle fibers (Boonsumrej, Chaiwanichsiri, Tantratian, Suzuki, & Takai, 2007; Kim et al., 2018). The characteristic freezing time (t_c), defined as the time to pass through the critical temperature zone (Anõn & Calvelo, 1980), has over the years been used to measure how the freezing rate affect ice crystals formation mainly based on histological analysis (Bevilacqua et al., 1979; Grujić et al., 1993; Martino, Otero, Sanz, & Zaritzky, 1998). The formation of extracellular ice is accompanied with an increase of solute concentration in the surrounding non-frozen water, potentially creating an osmotic pressure to cause the migration of water from inside to outside of the muscle fibers. Freezing at slow rate often forms large ice crystals distributed unevenly in the extracellular area. However, fast freezing could quickly remove more latent heat from the system than that produced from the extracellular nuclei formation, thereby potentially achieving the supercooling point required for intracellular nucleation. Thus, fast freezing often leads to numerous small ice crystals uniformly located within and between the muscle fibers. New visualization techniques to assess the size and distribution of ice crystals are constantly being developed as reviewed by Zhu et al. (2019), thus expanding our understanding of the characteristics of ice crystallization during freezing. Mulot, Fatou-Toutie, Benkhelifa, Pathier, & Flick (2019) reported more numerous smaller and rounder ice crystals distributed much narrower at higher freezing rates compared to slow, as imaged by X-ray micro-computed tomography.

2.1.3. Changes in structure and water-holding capacity of meat during freezing-thawing

When the temperature declines below -1 °C water in the extracellular area starts to freeze out, the amount being highly dependent on the subzero freezing temperature. The content of frozen water is around 75% of the total muscle water at -5 °C and 85% at -10 °C, and reaches above 90% at -20 °C (Calvelo, 1981; Grujić et al., 1993). As ice crystals grow in the extracellular space, solutes gradually become more concentrated in the surrounding non-frozen water (Ohta & Tanaka, 1978). A high ionic strength at -5 °C has by Finn (1932) been reported to correspond to 1.6 M potassium chloride. The

exposure of the muscle fibers to concentrating solutes in the extracellular area for a long duration during freezing would produce a higher extracellular osmotic pressure pulling water within the muscle fibers to flow outwards, possibly resulting in transversal shrinkage of the muscle fibers and a subsequent dehydration. Offer & Knight (1988b) has proposed that myofibrillar shrinkage during freezing due to this increased extracellular osmotic pressure could reach a limit set by electrostatic swelling pressure (Hamm, 1986) and osmotic pressure (Offer & Knight, 1988a) originally occurring within and between the filaments. Additionally, it is well known that ionic strength can significantly influence the structure within the myofibrils, and at an ionic strength above 0.25 M NaCl the myosin filaments are progressively dissociated to myosin molecules (Josephs & Harrington, 1966). The increased ionic strength in the unfrozen extracellular fluid in freezing could thus hypothetically depolymerize the myosin filaments causing an entropy swelling pressure to oppose the increased external osmotic pressure (Offer & Knight, 1988b). As ice crystals melt on thawing, the solute concentration in the unfrozen water gradually decrease and the external osmotic pressure will then decline, and therefore, muscle fibers hydrate and expand to recover much of their unfrozen volume (Ishiguro & Horimizu, 2008).

2.1.3.1. Effects of freezing-thawing on structure within meat tissue

The structure within the meat tissue changes on freezing-thawing in relation to myofibrillar shrinkage and subsequent cell dehydration following movement of water from inside to outside of muscle fibers during freezing as discussed above. Sigurgisladottir, Ingvarsdottir, Torrissen, Cardinal, & Hafsteinsson (2000) reported a decrease by around 20% of cross-sectional area within the muscle fibers accompanied with increased extracellular space in frozen-thawed salmon muscle. Also Sriket, Benjakul, Visessanguan, & Kijroongrojana (2007) observed more separated muscle fiber bundles and loss of Z-disk in shrimp muscle after being subjected to freeze-thaw cycles. Reduced fiber diameter within the beef tissue was observed by Martino & Zaritzky (1988) following a decrease in freezing temperature. In addition, a slight decrease of the distance between myosin filaments has been found by Jarenback & Liljemark (1975a) in cod muscle after freezing and thawing, and this decrease became more evident with prolonged frozen storage.

The freezing rate significantly affect the size and distribution of ice crystals thus causing changes of structure within the meat tissue. As suggested by Añón & Calvelo (1980) and Bevilacqua et al. (1979), fast freezing corresponding to t_c value < 15 min produces many small intracellular ice crystals, and

therefore, few distortions of muscle fibers and low exudate amounts upon thawing were observed. As t_c value increases, the number of these intracellular ice crystals would be expected to decrease with a simultaneous increase in size. This tendency continued until the t_c value reached around 20 min, and by then there was only a single intracellular crystal occupying each fiber, giving rise to mechanical damage thus explaining the observed largest amount of water loss upon thawing. With regard to longer t_c values, ice could be formed mainly in the extracellular area followed by the migration of intracellular water towards extracellular space causing partial dehydration of the muscle fibers. The effect of intracellular damage to muscle fibers would then be more or less excluded and consequently the amount of exudate loss was reduced but still larger than the amount observed at very small values of t_c. However, at thawing the phenomena run in opposite direction, the thawing time would be of importance: in fast thawing the water will melt in the extracellular area quickly creating a large thaw loss, but with a slow thawing there will be longer time for water to return to the fibers (Añón & Calvelo, 1980). More recently, Ishiguro & Horimizu (2008) investigated histological changes of tissue in fast and slow frozen-thawed chicken meat by using staining, and distinct differences of structure were observed between different freezing rates (Fig. 2). Unfrozen meat tissue showed compact and well-organized muscle fibers as well as connective tissues (Fig. 2a, a', and a''). Slow freezing and fast thawing produced both intracellular and extracellular freezing-induced damage to muscle structure (Fig. 2b, b', and b''). In the extracellular area, some large cracks between the muscle fibers were observed following shrinkage of the fibers, while connective tissues in those cracks had separated from the fibers. Additionally, a few large cracks due to the formation of intracellular ice crystals were found locally within the muscle fibers. Fast freezing and fast thawing achieved much less deformation of muscle fibers compared to slow freezing, but more evident effects of intracellular freezing were shown by the appearance of numerous small and fine cracks in the muscle fibers (Fig. 2c, c', and c''). Also some narrower cracks between fibers were observed corresponding to extracellular freezing-induced damage occurring in fast freezing. In agreement, increased spacing between the fiber bundles has by Chen & Pan (1997) been reported to be accompanied with decreased freezing rates in tilapia muscle, and by Grujić et al. (1993) in beef.

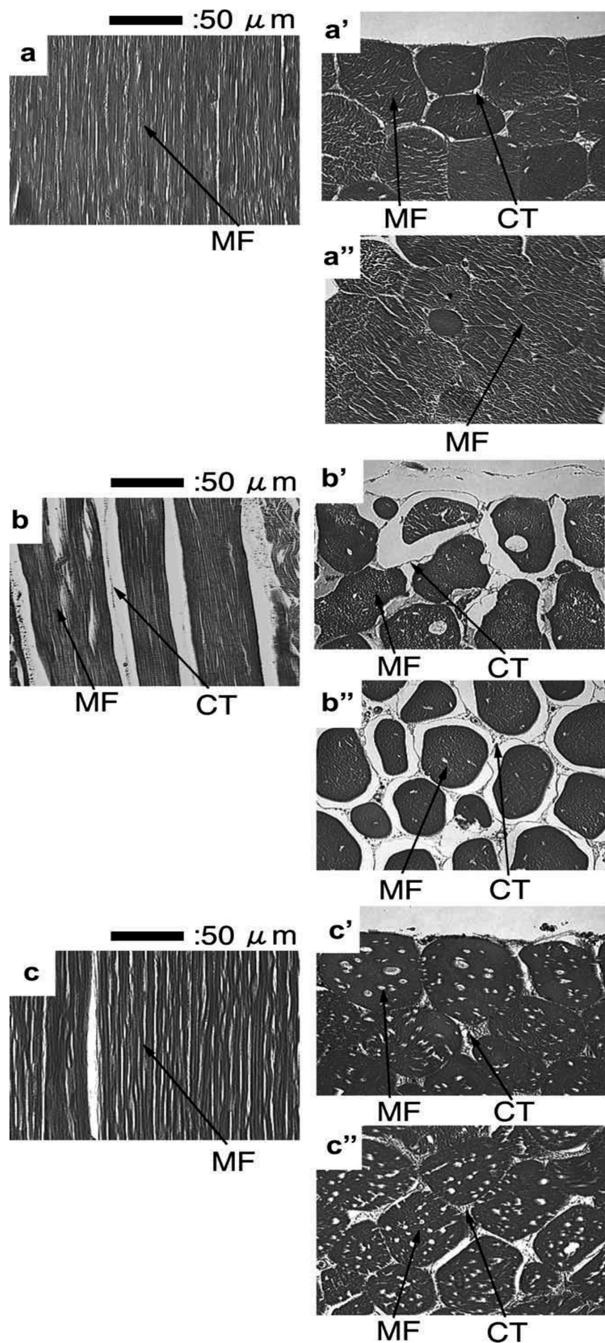

Fig. 2. Histological change of chicken meat tissues after freezing-thawing. (a) Control, (b) Slow freezing and fast thawing, (c) Fast freezing and fast thawing. a-c: The horizontal images parallel to the muscle fibers direction. a', a'', b', b'', c' and c'': The perpendicular section images to the muscle fibers. a'-c' and a''-c'': Images are taken near the surface and inside of the sample, respectively. MF: muscle fibers. CT: connective tissues. (Ishiguro & Horimizu, 2008)

2.1.3.2. Effects of freezing-thawing on water-holding capacity of meat

Freshly cut muscle contains around 75% water, and majority of which is the entrapped water held within the myofibrils (Huff-Lonergan & Lonergan, 2005). It is generally accepted that freezing and thawing negatively influence WHC of meat often evaluated as thaw loss, drip loss and cooking loss (Aroeira et al., 2016; Lagerstedt, Enfält, Johansson, & Lundström, 2008; Leygonie et al., 2012a; Zhang & Ertbjerg, 2019). For example, non-frozen beef loins stored for 3 weeks produced around 3% of drip loss, whereas paired steaks that were frozen for 2 weeks produced on average 6% of water loss upon thawing (Kim & Kim, 2016). Repeated freezing-thawing cycles could cause more water to be released from the muscle structure (Benjakul & Bauer, 2000), reaching a limit of around 30% loss after 5 cycles (Cheng et al., 2019). Several studies focused on the freezing rate have shown that slow freezing often produces a higher thaw loss than fast freezing (Añón & Calvelo, 1980; Kim et al., 2018; Kim, Liesse, Kemp, & Balan, 2015; Ngapo et al., 1999). Recently, low-field NMR relaxation measurement has been applied to monitor changes of water populations in relation to WHC of meat caused by freezing and thawing. The mobility and population of the entrapped water have by Cheng et al. (2019) been observed to significantly decrease with an increased number of freeze-thaw cycles in beef *semimembranous* muscle, and by Ali, Zhang, Rajput, Khan, Li, & Zhou (2015) in chicken breast meat, potentially indicating a shift of the entrapped water to free water in freezing-thawing which would consequently lead to a weaker WHC in frozen-thawed meat. This result might be attributed to the migration of intracellular water to the extracellular areas during freezing after which part of the water would stay extracellular upon thawing and could not be reabsorbed by the muscle fibers (Sanchez-Alonso, Moreno, & Careche, 2014).

The cooking loss due to freezing and thawing has been reported by Grayson et al. (2014) to increase significantly in beef *longissimus lumborum* muscle, and by Choi, Park, Chung, Park, Kim, & Chun (2017) in pork loin. However, there is a lack of consensus in literature on the effects of freezing and thawing on cooking loss. Some studies did not find any differences of water loss due to cooking between fresh and frozen-thawed meat samples (Leygonie, Britz, & Hoffman, 2012b) as well as for samples frozen at different rates (Kim et al., 2018; Kim, Meyers, Kim, Liceaga, & Lemenager, 2017), and these authors attributed it to the substantial increase of water loss upon thawing.

2.2. Protein denaturation that occurs during freezing and thawing

2.2.1. Evidence of freezing-induced protein denaturation

Within meat tissue, protein is regarded as the second largest component accounting for around 20% following water (Lawrie, 1998). Meat proteins are generally found in the insoluble fraction (myofibrillar proteins and extracellular matrix proteins) or the soluble fraction (sarcoplasmic proteins). Myofibrillar proteins, comprising 60-70% of the total muscle proteins and mainly consisting of 45-50% hydrophobic amino acids and 25-30% hydrophilic amino acids (Hamm, 1972), build up a fibrous structure mainly assembled by thick and thin filaments (Offer & Knight, 1988a). Myosin accounts for 43% being the most abundant protein among the total myofibrillar proteins. The thick filaments are composed principally of the myosin molecule as a basic unit. In striated muscles, myosin consists of two head domains connected to a neck (heavy meromyosin) attached at one end of a tail (light meromyosin). The neck and tail both comprise two polypeptide chains coiled around each other into α-helical coiled-coil conformation. The polypeptide chains diverge at the head-neck junction, each folding into a globular structure at the head of myosin molecule which contains the enzymatic ATPase catalytic site and the actin-binding site (Rayment & Holden, 1994). The native structure of myosin is thus made up of a mixture of globular and rodlike fractions (Woods, Himmelfarb, & Harrington, 1963). Actin is the main component of the thin filaments and represents 22% of the total myofibrillar proteins. The fibrous actin (F-actin) is generated based on longitudinal polymerization of globular G-actin. Sarcoplasmic proteins are mainly present within the myofibrillar structure (Offer & Knight, 1988a) consisting of 45-50% ionic hydrophilic amino acids and 25-30% hydrophobic amino acids.

The native protein conformation is generally stabilized dependent on covalent forces e.g. disulfide linkage and non-covalent forces e.g. hydrogen bonds, hydrophobic interactions, electrostatic interaction, and van der Waals forces. Protein denaturation is a complex phenomenon often describing changes of the secondary and tertiary structures principally resulting from rupture of the non-covalent forces (Tanford, 1968). The idea that muscle undergoes protein denaturation induced by freezing and thawing was proposed more than half a century ago (Hamm, 1961), but some initial studies seem not to observe an irreversible denaturation of proteins due to the process itself. However, scientists have over the years tried to prove the phenomenon of freezing-induced protein denaturation with various analytical parameters. The denaturation occurring in muscle proteins during freezing and thawing is manifested as changes of protein in some physical and chemical characteristics. Evidence from

different animals and muscles showing that the relationship between freezing-thawing and protein denaturation corresponding to myofibrillar and sarcoplasmic proteins is respectively summarized in Table 2 and Table 3.

Table 2. Summary of evidence from literature indicating myofibrillar protein denaturation occurring during freezing and thawing.

Species	Freezing conditions	Denaturation evidence	Reference
Pork LTL	Frozen for two weeks then thawed overnight	Denaturation enthalpy ↓ Peak temperature ↓ Surface hydrophobicity ↑ WHC capaciy of myofibrils ↓	Zhang & Ertbjerg (2019)
Beef LTL	Frozen for 7 days then thawed at 4 °C	Protein solubility ↓ Surface hydrophobicity ↑ α-helix ↓ Ionic and hydrogen bonds ↓	Qian et al. (2019)
Pork tenderloin	Freezing then thawed immediately	Peak temperature ↓ Particle sizes ↑ Fluorescence intensity ↓	Jia, Nirasawa, Ji, Luo, & Liu (2018)
Pork LTL	Frozen for a week then thawed overnight	WHC capaciy of myofibrils ↓	Zhang & Ertbjerg (2018)
Pork tenderloin	Freezing then thawed under 20 °C for 1 h	Peak temperature ↓ Denaturation enthalpy ↓	Jia, He, Nirasawa, Tatsumi, Liu, & Liu (2017)
Pork LTL	Frozen for 7 days then thawed at 4 °C for 12 h	Dityrosine ↑ α-helix ↓ fluorescence emission wavelength ↑ UV second derivative spectra ↑	Zhang, Li, Diao, Kong, & Xia (2017)
Chicken breast	Frozen for a week then thawed for 12 h	Denaturation enthalpy ↓	Ali et al. (2015)
Lamb LTL	Frozen for 12 h then thawed for 12 h	Protein solubility ↓	Qi et al. (2012)

(Continuing next page)

Table 2. (Continued)

Species	Freezing conditions	Denaturation evidence	Reference
Turkey breast	Frozen for 3 weeks then thawed overnight	Ca^{2+}-ATPase activity ↓ Protein solubility ↓ Total sulfhydryl content ↓	Chan et al. (2011)
Pork LTL	Frozen for 4 days then thawed using running water	Denaturation enthalpy ↓ Peak temperature ↓ Surface hydrophobicity ↑ Emulsifying activity ↓	Xia, Kong, Xiong, & Ren (2010)
Pork LTL	Frozen for 4 days then thawed using tap water	Total sulphydryl content ↓ ATPase activity ↓	Xia, Kong, Liu, & Liu (2009)
Lamb LTL	Frozen for 1 day then thawed for 16 h	The SDS-PAGE bands (30- and 32-kDa) intensity ↑	Ojeda, Wagner, & Crupkin (2001)
Beef LTL	Frozen for 2-3 days then thawed for 7 h	Water-binding capacity ↓	Petrović, Grujić, & Petrović (1993)
Beef SD	Frozen until reaching -25 °C then thawed overnight	Denaturation enthalpy ↓ ATPase activity ↓	Wagner & Añón (1985)
Beef round	Frozen for 2 weeks then thawed	Actomyosin solubility ↓	Awad, Powrie, & Fennema (1968)
Chicken PM and BF	Frozen then thawed immediately	ATPase activity ↓ Sulfhydryl groups →	Khan & van den Berg (1967)

LTL *M. Longissimus thoracis et lumborum*; SD *M. semitendinosus*; PM *M. pectoralis major*; BF *M. biceps femoris*. '↑', increase; '↓', decrease; '→' no significant effect

Table 3 Summary of evidence from literature indicating sarcoplasmic protein denaturation occurring during freezing and thawing.

Species	Freezing conditions	Denaturation evidence	Reference
Pork LTL	Frozen for two weeks then thawed overnight	Denaturation enthalpy ↓ Fluorescence intensity ↑	Zhang & Ertbjerg (2019)
Chicken breast	Frozen for a week then thawed for 12 h	Zeta-potential of exudate↓	Chen et al. (2017)
Lamb LTL	Frozen for 12 h then thawed for 12 h	Protein solubility ↓	Qi et al. (2012)
Turkey breast	Frozen for 3 weeks then thawed overnight	Protein solubility ↑ Surface hydrophobicity↓	Chan et al. (2011)
Pork LTL	Frozen for 3 days then thawed for 11 h	Protein band (97kDa) intensity by electrophoresis↓	Hansen, Trinderup, Hviid, Darré, & Skibsted (2003)
Pork LTL	Frozen then thawed within 7 days	Protein concentration in drip↓	Penny (1975)
Beef round	Frozen for 2 weeks then thawed	Protein solubility↓ Protein content in drip↑ Insolubilization in electrophoresis ↑	Awad et al. (1968)

LTL *M. longissimus thoracis et lumborum*

'↑', increase; '↓', decrease; '⟶' no significant effect

Solubility and extractability

The most popular measurement used to investigate the freezing-induced protein changes are related to loss in the solubility or extractability of meat proteins in early studies (Finn, 1932; Miller, Ackerman, & Palumbo, 1980; Snow, 1950). It was well manifested by many authors that myofibrillar proteins in freezing-thawing often undergo a significant decline in solubility or extractability, thus contributing to increased loss in total protein solubility (Awad et al., 1968; Chan et al., 2011; Qi et al., 2012; Qian et al., 2019). Sarcoplasmic protein solubility was observed to reduce in frozen-thawed lamb (Penny, 1975)

and beef (Awad et al., 1968) as compared to fresh. However, a distinct increase ($P < 0.05$) has been reported by Chan et al. (2011) in turkey breast meat after freezing and thawing.

Surface hydrophobicity

Surface hydrophobicity has often been used to indicate protein denaturation occurring during freezing and thawing of muscle food (Xiong, 1997). The polar amino acid side chains on proteins are generally orientated outwards close to the water within the fibers and the non-polar groups inwards. On the myosin filaments the surface is more hydrophilic, whereas the inner core presents as a hollow structure which contains more hydrophobic groups. Protein unfolding or rupture of the hydrophobic core in the myosin filaments often cause an exposure of inner hydrophobic groups to the surface, thus contributing to surface hydrophobicity (Lin & Park, 1998).

The most common methodologies to determine alterations of protein surface hydrophobicity are to measure the fluorescence of compounds e.g. 1-anilino-8-naphthalene-sulphonate (ANS) (Chan et al., 2011) and cis-parinaric acid (CPA) (Kato & Nakai, 1980) since they are able to identify the hydrophobic sites exposed on the surface of proteins. An increase of fluorescence often manifests as a more hydrophobic environment following the exposure of inner hydrophobic groups occurring in the protein structure. Several studies have focused on myofibrillar proteins and found that freezing and thawing significantly increased surface hydrophobicity (Qian et al., 2019; Xia et al., 2010; Zhang & Ertbjerg, 2019). Regarding sarcoplasmic proteins, however, Chan et al. (2011) found decreased surface hydrophobicity and they attributed it to reduced exposure of hydrophobic groups on the surface since soluble proteins possibly became more folded or aggregated during freezing and thawing. Nevertheless, these fluorescent compounds are often applicable to test protein solutions thus causing a limitation when applied to meat proteins particularly myofibrillar proteins due to their low solubility. Pre-solubilization of these structural proteins in strong salt solutions could alter their biochemical properties and thus create artifacts into the determinations. Bromophenol blue (BPB) has been developed by Chelh, Gatellier, & Santé-Lhoutellier (2006) to determine surface hydrophobicity of structural proteins based on the interaction of BPB with hydrophobic sites on myofibrillar proteins. Other works (Liu, Arner, Puolanne, & Ertbjerg, 2016; Liu, Puolanne, & Ertbjerg, 2014) have confirmed this determination on surface hydrophobicity in the myofibrils incubated from 20 to 40 °C.

Differential scanning calorimetry (DSC)

Thermal stability of protein structure has extensively been investigated by differential scanning calorimetry (DSC) in muscle foods, especially in cooked meat products (Martens, Stabursvik, & Martens, 1982). Three major endothermic peak transitions have often been detected in the DSC thermogram to indicate protein denaturation patterns corresponding to alterations of peak temperature and denaturation enthalpy (Fig. 3). The first peak in the DSC thermogram has been ascribed to myosin denaturation, the second peak was corresponded to sarcoplasmic proteins and collagen denaturation, and the third peak has been assigned to actin (Stabursvik & Martens, 1980; Xiong, Brekke, & Leung, 1987). Many studies have found that freezing and thawing could decrease significantly the thermal stability within the myosin structure in different animals shown as reduced myosin peak temperature (Jia et al., 2017; Jia et al., 2018; Xia et al., 2010) or denaturation enthalpy (Jia et al., 2017; Wagner & Añón, 1985). Also a distinct loss of denaturation enthalpy was observed in the myofibrils isolated from frozen compared to fresh beef muscle (Wagner & Añón, 1985). When compared with myosin, actin and sarcoplasmic proteins are less sensitive with respect to freezing-induced thermal destabilization. Few studies reported alterations of the second and third peak in DSC thermograms (Jia et al., 2017; Xia et al., 2010).

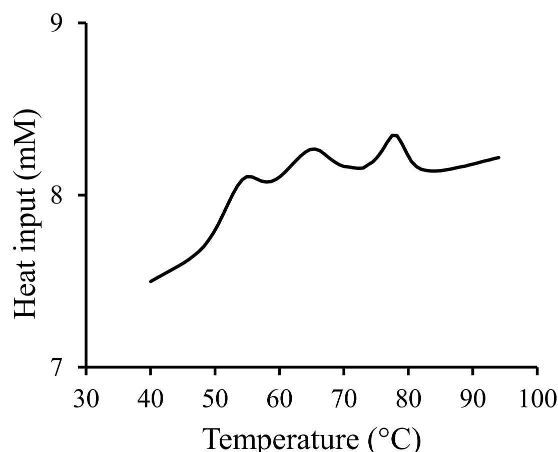

Fig. 3. Representative DSC thermogram for pork muscle consisted of three major endothermal peaks (Zhang & Ertbjerg, 2019).

Other indicators

The loss of ATPase activity has also been used to reveal the integrity of the myosin molecule thus indicating denaturation of myofibrillar proteins (Benjakul & Bauer, 2000). The myosin molecule has two globular heads and each myosin head contains one enzymatic ATPase active site which can catalyze ATP hydrolysis transforming chemical to mechanical energy for muscle contraction (Rayment & Holden, 1994). Any structural alterations in the myosin head due to external conditions such as freezing-thawing could negatively influence the recognition of ATP molecules thus contributing to a loss of ATPase activity. Therefore, the myosin molecule is expected to be susceptible to freezing-induced denaturation as suggested by a decline in Ca^{2+}-ATPase activity. Wagner & Añón (1985) found a decrease by 47% in Ca^{2+}-ATPase activity from frozen-thawed beef. Also Chan et al. (2011) reported a 35% loss of Ca^{2+}-ATPase activity in frozen-thawed turkey compared to fresh. Slight decreases of ATPase activity by around 20% have been found in pork (Xia et al., 2009) and poultry muscle (Khan & van den Berg, 1967) during the process of freezing and thawing.

Protein often contains amino acid residues such as tryptophan, tyrosine, and phenylalanine which present intrinsic fluorescence attributes, and any conformational changes in the protein structure could alter the fluorescence intensity and local mobility of these aromatic amino acids (Royer, 2006). Among these side chains, tryptophan residues have relatively high extinction coefficients and quantum yields, and their emission energy is often more sensitive to external environments. Thus, the fluorescence spectra of tryptophan residues are expected to estimate changes of protein tertiary structure in terms of the maximum emission wavelength (λ_{max}) and fluorescence intensity (FI_{max}). Using intrinsic emission fluorescence spectroscopy, Zhang et al. (2017) observed slight shift of λ_{max} to longer wavelengths and decreased FI_{max} in myofibrillar proteins with increased freezing and thawing cycles. Jia et al. (2018) found decreased FI_{max} in myofibrillar proteins extracted from frozen-thawed pork. These observations strongly suggest an exposure of the partially buried tryptophan to a polar environment related to myofibrillar protein unfolding occurring during freezing and thawing. UV derivative spectroscopy has also been used to detect freezing-induced changes of micro-environment within aromatic amino acids such as tryptophan, tyrosine and phenylalanine (Cao et al., 2018; Filgueras, Gatellier, Zambiazi, & Santé-Lhoutellier, 2011; Zhang et al., 2017), potentially verifying myofibrillar protein unfolding during freezing and thawing. New nondestructive techniques to evaluate the degree of freeze damage to meat proteins are constantly being developed. Egelandsdal et al. (2019)

has suggested a role of microwave spectroscopy in monitoring the changes of dielectric properties within meat proteins resulting from protein denaturation and aggregation occurring during freezing and thawing.

2.2.2. Traditional theories to explain mechanisms behind freezing-induced protein denaturation

Freezing of muscle mainly involves changes of water phase within meat tissue from an amorphous state to highly structured ice crystals. The resultant alterations in the physical and chemical environment within the muscle protein could contribute to protein denaturation. The conventional mechanisms to explain freezing-induced protein denaturation were recognized in early reviews (Mackie, 1993; Shenouda, 1980; Xiong, 1997) mainly based on the mechanical damage due to ice crystals formation and also the effect of an increase in solute concentration in the unfrozen water.

2.2.2.1. Mechanical damage to protein structure due to ice crystal formation

Protein conformation generally follows the pattern in which a large fraction of hydrophobic amino acids is buried inside and hydrophilic or charged groups tend to stay on the protein surface. This structure thus facilitates the formation of hydrogen bonds contributing to hydrophilic interaction between surface protein and water molecules. In most native proteins, however, some hydrophobic groups e.g. leucine, valine and alanine are exposed on the outer surface of the myosin filament. The surrounding water molecules could adhere to these hydrophobic groups possibly forming water-mediated hydrophobic-hydrophilic linkages (Lewin, 1974). Moreover, hydrophobic interactions of protein molecules are also based on the strong cohesion of hydrated water molecules since this cohesion could expel the hydrophobic groups from water (Puolanne & Halonen, 2010). Thus, water molecules within muscle tissue play an important role in maintaining the network of hydrogen bonds and hydrophobic interactions in the three-dimensional protein conformation.

It has been well recognized that freezing, especially at a slow rate, brings about ice crystallization at the expense of water molecules in the intra and extracellular areas within muscle tissue thus causing damage to muscle structure and dehydration of protein molecules. In the dehydrated state, a disruption of the hydrogen bond network and an increased exposure of hydrophobic and hydrophilic groups are expected to occur due to the rupture of the organized water structure, thus leaving these regions more unprotected. Consequently, more hydrophobic interactions between protein molecules would then be

expected to take place, destabilizing the protein three-dimensional structure, thus contributing to denaturation and the resultant aggregation. Myofibrillar protein denaturation as indicated by decreased hydrogen bonds coupled with increased surface hydrophobicity has been reported by Qian et al. (2019) in frozen-thawed beef muscle.

2.2.2.2. Increase in solutes concentration in the unfrozen water

An increase in ionic strength has often been regarded as one of earliest theories to explain freezing-induced protein denaturation. Water starts to be frozen out below -1 °C, and a crystallization of a higher percentage of the tissue water would thus take place as the temperature declines, but a considerable portion may still be present in the unfrozen state (Calvelo, 1981). As freezing progresses, solutes will thus become progressively more concentrated in the remaining unfrozen water, possibly giving it a high ionic strength at -15 °C corresponding to around 3 M potassium chloride (Finn, 1932). This might influence the electrostatic interactions within the protein native structure, consequently inducing protein denaturation. Increased ionic bonds as well as decreased hydrogen bonds have been reported by Jiang et al. (2019a) in lightly salted tuna. Salts at comparatively low concentrations often have a solubilizing effect on protein molecules. For example, thick filaments are progressively dissociated into myosin molecules at a concentration of 0.25 M NaCl (Josephs & Harrington, 1966). At very high ionic strength, above 1.5, salting-out may occur to reduce the amount of hydrophilic groups associated with water molecules thus leading to hydrophobic association and precipitation of proteins (Nakai and Li-Chan, 1988). Myosin denaturation has been found by Duerr & Dyer (1952) and Lin & Park (1998) in cod and salmon when being exposed to ionic strength above 1.5, as indicated by decreased protein solubility. These authors attributed it to partial protein unfolding caused by the rupture of ionic linkage and subsequent aggregation of exposed hydrophobic groups. The thermal stability of myosin and actin was also observed to decrease during salting of cod, indicating a role of salt in destabilizing protein conformation (Thorarinsdottir, Arason, Geirsdottir, Bogason, & Kristbergsson, 2002).

2.2.3. The importance of freezing temperature at around -3 °C on rate of protein denaturation

The rate of protein denaturation is highly dependent on the freezing temperature, often becoming progressively decreased as the freezing temperature is reduced (Buttkus, 1970; Hiner, Madsen, & Hankins, 1945). However, it has been well accepted that concentrating solutes in the unfrozen water

during freezing is responsible for protein denaturation as discussed above (Xiong, 1997; Sikorski, Olley, Kostuch, & Olcott, 1976), and in general, the lower the freezing temperature, the higher the solute concentration (Calvelo, 1981). The temperature from -1 to -7 °C has been recognized as the critical zone where t_c of freezable water is frozen together with concentrating solutes in the unfrozen water thus leading to an accelerated rate of biochemical and enzymatic reactions within the meat tissue. Moriya, Nakazawa, Osako, & Okazaki (2019) observed increased ATP depletion rate when frozen chub mackerel was kept at -2 °C prior to thawing. The importance of the freezing temperature on the rate of protein denaturation is thus to be expected, and the maximum rate of denaturation has often been proposed to occur at a temperature within the critical temperature zone in early studies. Calvelo (1981) and Finn (1932) both recognized this maximum rate existed at -3 °C in beef. However, this temperature could vary slightly between species. The rate of the protein insolubilization reaction in frozen cod has early been reported by Love (1962) and Love & Elerian (1964) to be maximal at around -1.5 °C.

Several studies have found a severe protein denaturation occurring at the freezing temperature of around -3 °C. Around 32% loss of total extractable proteins in beef was reported by Awad et al. (1968) during storage at -4 °C for two weeks. Also Sun, Zhao, Ling, Yu, Shang, & Liu (2017) found a decrease by 30% of protein extractability and nearly 50% loss of Ca^{2+}-ATPase activity when swimming crab was kept at -3 °C for 15 days. Kaale & Eikevik (2016) reported around 10% loss of protein stability in salmon when kept at -1.7 °C for 3 days, being comparable with that observed in deep freezing for 30 days. These observations give direct evidence of protein denaturation occurring at around -3 °C. When freezing of water take places, the latent energy of ice crystallization can be removed and then there will be an instantaneous rise of the temperature to equilibrium temperature. Rahman, Kasapis, Guizani, & Al-Amri (2003) has observed a slight increase of temperature as the freezing temperature declined to around -3 °C during freezing of tuna meat. Under industrial thawing, blocks of frozen ground meat often need a long time to be completely thawed considering a large quantity often stored in the industry. The meat temperature during thawing would probably remain at around -3 °C for a long duration due to the high latent heat within this temperature zone, thus leading to a high probability of accelerated protein denaturation occurring with prolonged thawing time. More research needs to be focus on the effect of the industrial thawing on protein characteristics in relation to meat quality.

2.3. Protein denaturation in relation to water-holding capacity of meat

2.3.1. Effect of structural alterations within meat tissue related to denaturation

Myofibrils generally trap a major part of water within meat tissue, and WHC of meat is to a large extent dependent on the properties of the myofibrils, particularly shown in changes of the myofibrillar volume or more precisely, unit cell volume, i.e. sarcomere length × lattice area (Offer & Knight, 1988a). Factors that can induce lateral shrinkage within the myofibrillar structure could induce water loss from meat (Hughes, Oiseth, Purslow, Warner, 2014; Offer & Trinick, 1983). Myofibrillar proteins thus play an important role in regulating the water within the muscle structure, and any physicochemical changes occurring in the myofibrillar proteins might strongly be linked to the WHC of meat (Penny, 1969).

The combination of temperature and pH i.e. high temperature and low pH in the case of pale, soft and exudative (PSE) meat will induce denaturation within myofibrillar proteins, which has been well recognized as the main mechanism for the extensive loss of WHC (Kim, Warner, and Rosenvold, 2014; Pietrzak, Greaser, & Sosnicki, 1997; Warner, Kauffman, & Greaser, 1997). Also, it has by Offer & Knight (1988b) and Zhu, Ruusunen, Gusella, Zhou, & Puolanne (2011) been hypothesized that myosin denaturation could cause myofibrillar shrinkage thus potentially accounting for the water loss found in PSE state. The structural alterations within myosin molecules in PSE meat, as indicated by reduced ATPase activity, could possibly change the shape of the cross-bridges i.e. a shortening in the length of myosin molecules thus contributing to the decreased distance between thick and thin filaments and the subsequent shrinkage within the whole myofibrillar structure. Walker & Trinick (1986) reported a reduction by around 20 nm in length of myosin molecules occurring during incubation at around 40 °C. As observed by Liu et al. (2016), incubation at 44 °C to simulate PSE condition reduced significantly the lattice spacing between the filaments by around 10% as compared to incubation at 21 °C. Consequently, water would then flow into the enlarged extracellular space between the fibers where it can easily be lost afterwards (Swatland, Irving & Millman, 1989). The denatured sarcoplasmic proteins precipitated onto myofibrils has also been proposed to influence the WHC in PSE meat (Bowker & Zhuang, 2015; Liu et al., 2016).

Accordingly, at low temperature i.e. freezing temperature, the observation in frozen-thawed muscle food of the shrinkage in the muscle fibers together with decreased distance between thick filaments (Jarenback & Liljemark, 1975a; Martino & Zaritzky, 1988) as well as enlarged extracellular space (Sigurgisladottir et al., 2000) would correspond to the structural alterations within myofibrillar proteins

potentially attributed to the migration of water from intra- to extracellular areas as described in section 2.1.3 as well as to the freezing-induced protein denaturation. As shown by Jarenback & Liljemark (1975b), freezing at -10 °C caused a reduction in the length and number of attached myosin molecules in the extracted actomyosin filaments. Also the ATPase activity and DSC measurement in the section 2.2.1 clearly suggested that conformational changes occurred in the myosin area due to freezing-thawing. With regard to the characteristics of WHC of meat, the main explanation found in PSE meat is the denaturation of myosin causing the increased myofilamental shrinkage and consequently reduced WHC of meat as discussed above. Likewise, the structural alterations within the myofibrillar structure occurring in freezing-thawing might thus potentially indicate a role of freezing-induced denaturation in reduced WHC of meat upon thawing.

2.3.2. Roles of pH and ionic strength

The surface polarity within the myofilaments remains negative at about pH 5.5 and the relatively low ionic strength in postmortem meat. Myofibrillar proteins often play an important role in controlling WHC, and as hypothesized by Hamm (1972), the electrostatic force between the myofilaments are highly responsible for the changes of the myofibrillar volume consequently affecting the amount of water that can be held within the myofibrils. It has been well demonstrated that pH and ionic strength greatly contribute to WHC within meat tissue (Offer & Knight, 1988a; Puolanne & Halonen, 2010). The effect of pH and ionic strength on WHC has been ascribed to the denaturing effect on the electrostatic force between the charged myofilaments accompanied with the subsequent changes within the myofibrillar volume (Hamm, 1986). Typical influence of pH and NaCl is illustrated in Fig. 4. The average isoelectric point (p*I*) of myofibrillar proteins is close to pH 5.0, where the positive charges are equal to the negative charges thus causing minimum net charges on myofilaments as well as minimum myofilamental space. A shift of pH towards the acidic or basic side would respectively lead to more positive or negative net charges of the myofibrillar proteins, thereby contributing to enlarged myofilamental distance (Fig. 4) and better WHC. The contribution of ionic strength to myofibrillar structure closely relates to pH. Chloride ions (rather than Na^+) has been proposed to be preferably bound to myofilaments, and as shown in Fig. 4, the addition of NaCl increases the electrostatic repulsion between the myofilaments at pH above the p*I*, while it has a reducing effect at pH below the p*I*.

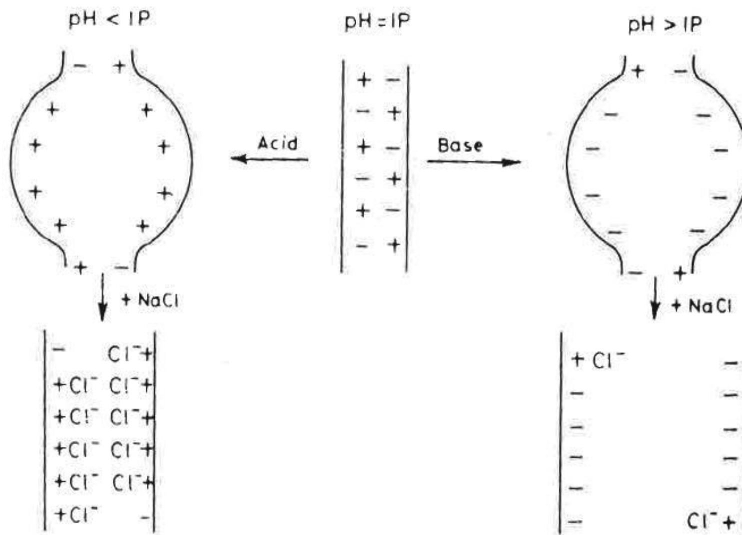

Fig. 4. Scheme on the effect of the combination of pH and NaCl on swelling between two adjacent myosin filaments (p*I*, isoelectric point) as described by Hamm (1986).

As reviewed by Puolanne & Halonen (2010), added salts would produce an effect on net charges within myosin filaments with regard to amino acids composition. Na^{+} could interact with the negatively charged amino acids e.g. aspartic acid and glutamic acid on the side chains of myosin filaments, while Cl^{-} can easily be absorbed by the positively charged groups e.g. arginine, histidine, and lysine on the outer surface. In addition, chloride ions as anionic chaotrope could also be absorbed to the hollow and hydrophobic core within the myosin filaments. Thus, the added salts are expected to increase the negative charges on the myofilaments in postmortem meat with an ultimate pH at around 5.5, thereby enlarging the electrostatic repulsion and introducing swelling of the myofibrillar systems. Chantarasuwan, Benjakul, & Visessanguan (2011) observed decreased Zeta potential values in natural actomyosin of Pacific white shrimp when treated with 2.5% NaCl, suggesting increased negative charges on the surface of actomyosin. Moreover, Knight & Parsons (1988) found that NaCl concentration up to 3 M could introduce swelling occurring in the isolated myofibrils, thus potentially contributing to better WHC. Also Puolanne & Peltonen (2013) observed increased WHC of postmortem meat with pH 5.5 when treated at ionic strength ranging from 0.5 to 1.5. Moreover, the addition of 2% NaCl in comminuted beef changed the p*I* of myofibrillar proteins to pH 4.0 due to a

preferred binding of negative charged Cl^- ions to myosin filaments, and therefore, increased WHC was observed when the pH of meat was around 5.5 (Hamm, 1986).

Increased ionic strength would be expected to decrease interactions within myofibrillar proteins (Wu & Smith, 1987), and thus affect the protein solubility within meat products as discussed in section 2.2.2.2. As demonstrated by Puolanne & Halonen (2010), the absorption of chloride ions to the hydrophobic core of myosin filaments would potentially rupture the inner core and then expose the inner hydrophobic groups to water thus possibly promoting the dissolution of myosin filaments into the molecules. Simultaneously, a disruption of electrostatic linkages caused by the changes of the charges on the protein surface would also lead to conformational changes possibly favoring hydrophobic interactions within protein molecules (Lin & Park, 1998). The exposure of hydrophobic groups that occurs in the myosin filaments caused by elevated ionic strength thus potentially results in protein denaturation, but it conversely contributes to better WHC as described above.

The pH in meat tissue has been recognized by Puolanne & Peltonen (2013) as a relatively more important variable than ionic strength, and they observed a larger effect on WHC caused by changing the pH from 5.4 to 4.8 rather than the ionic strength from 0.5 to 1.5. The contribution of pH to myofibrillar structure and WHC is significant as shown in Fig. 4, and a decrease of postmortem meat pH towards the acidic side would undoubtedly reduce the number of negative charges on the myofilaments (Hamm, 1986), recently indicated by increased Zeta potential values (Shen, Zhao, & Sun, 2019). Moreover, reducing pH has been recognized to strongly denature myofibrillar proteins as indicated by lower protein solubility and higher surface hydrophobicity (Sharedeh, Gatellier, Astruc, & Daudin, 2015; Van Laack & Lane, 2000), possibly leading to conformational changes within myofilaments and consequently affect the negative charges on the protein surface.

It has been well recognized that freezing and thawing could cause reduced WHC of meat as well as protein denaturation (sections 2.1.3.2 and 2.1.1). Increased solutes concentration in the unfrozen water has traditionally been regarded as the main theory to explain the freezing-induced protein denaturation (section 2.2.2.2). Myofibrillar proteins generally interact with water molecules consequently influencing WHC within the meat structure. In Study II (Zhang & Ertbjerg, 2019), denaturation of myofibrillar proteins occurring in freezing has been proposed to be directly linked to the decreased WHC in the myofibrils isolated from frozen-thawed meat. Elevated ionic strength would increase the electrostatic repulsion between the myofilaments thus causing better WHC as discussed above.

However, salting before freezing has been shown to reduce the microstructural damages occurring during freezing as well as to maintain better WHC and texture properties in freezing-thawing cycles (Jiang et al., 2019a; Jiang, Jia, Nakazawa, Hu, Osako, & Okazaki, 2019b). Thus it can be questioned if increased solute concentration in the unfrozen water is an absolute causative factor in explaining the mechanism of freezing-induced protein denaturation.

The combinations of decreased pH and elevated ionic strength have been found to cause decreased WHC of meat tissue (Puolanne & Peltonen, 2013) and of the isolated myofibrils (Zhang & Ertbjerg, 2019). More than 90% of the water will be frozen out below -20 °C, theoretically resulting in a > 10-fold increase of solutes and proton concentration in the unfrozen liquid. Thus the involvement of decreased pH is to be expected to cause reduced negative charges within myofilaments inducing protein denaturation and consequently loss of WHC, being comparable to the observation in frozen-thawed meat. In agreement, Chen et al. (2017) found increased Zeta potential values in chicken breast following freezing-thawing cycles, possibly indicating reduced negative charges on the protein surface during freezing-thawing. In addition, the structural alterations observed in frozen-thawed meat that muscle fibers shrunk accompanied with decreased spaces between thick filaments (section 2.1.3.1) seem also to support reduced electrostatic repulsion between filaments in relation to pH decline.

2.4. Hypotheses for causes of thaw loss

2.4.1. Current explanation based on ice crystal formation and water transfer

The accepted explanation for the formation of thaw loss in literature has often been made directly on the ice crystallization during freezing and the resultant mechanical damage to the muscle fiber structure inducing the loss of WHC (Añón & Calvelo, 1980; Hamm, 1986; Li et al., 2018; Zhu et al., 2019). The transfer of water within muscle tissue is to be expected during freezing-thawing in relation to the size and distribution of ice crystals that form inside or outside of the muscle fibers dependent on the freezing rate as described in section 2.1.2. Slow freezing often produces large extracellular ice crystals located unevenly between the muscle fibers following the migration of water from inside of muscle fibers to outside (Bevilacqua et al., 1979; Grujić et al., 1993). Fast freezing, however, causes the formation of small ice crystals distributed uniformly in the intra- and extracellular spaces within the muscle structure (Bevilacqua & Zaritzky, 1980; Kim et al., 2018). It is well established that slow freezing could generate more thaw loss as compared to fast freezing (Calvelo, 1981; Hamm, 1986), and many studies attributed

it to the differences in size and location of ice crystals in relation to different freezing rates (Nusbaum, Sebranek, Topel, & Rust, 1983; Sakata, Oshida, Morita, & Nagata, 1995). Additionally, larger mechanical damage shown as a rupture of muscle fibers potentially caused by large extracellular ice crystals in slow freezing is also believed to negatively affect the reabsorption of water by muscle fibers upon thawing, as compared to smaller damage caused by small ice crystals formed in fast freezing (Hamm, 1986).

2.4.2. Role of myofibrillar protein denaturation

Freezing and thawing could induce protein denaturation occurring in muscle food. Myofibrillar proteins account for 60-70% of the total muscle proteins and build up the myofibrillar structure which occupies the largest volume within the muscle fibers and thus traps the majority of the muscle water (Huff-Lonergan & Lonergan, 2005). Therefore, any physicochemical alterations of myofibrillar proteins could be linked to a reduction in the amount of water held by the myofibrillar structure potentially contributing to the formation of thaw loss. Nonetheless, as reviewed by Hamm (1986) and Leygonie et al. (2012a), the contribution of freezing-induced myofibrillar protein denaturation to the decrease in WHC of meat upon thawing has been estimated as small since several initial studies seemed not to observe an excessive denaturation within myofibrillar proteins induced by the freezing-thawing process itself. New measurement techniques have over the years been developed to present evidence for the freezing damage to myofibrillar proteins as discussed in section 2.2.1, thus expending our understanding in the role of myofibrillar protein denaturation in the generation of thaw loss.

The rate of freezing strongly influences the amount of thaw drip and that slow freezing often produce more thaw loss than fast freezing. Some studies on freezing rate did not observe a distinct influence on protein denaturation (Farouk, et al., 2003; Ngapo, et al., 1999). The current explanation for the different amounts of thaw drip caused by different freezing rates is mainly in relation to the size and location of ice crystals. It was, however, noted by other studies that slow freezing resulted in a more pronounced denaturation occurring in the myofibrillar proteins compared to fast freezing, and evidence investigated by different methodologies from different animals and muscles is summarized in Table 4. Nevertheless, the role of myofibrillar protein denaturation in the generation of thaw loss remains not well understood, and more research is required to evaluate the relative importance of protein denaturation.

Table 4. Summary of evidence from literature indicating more pronounced myofibrillar protein denaturation that occurred in slow than fast freezing.

Denaturation indicator	**Species**	**Reference**
Protein solubility	Beef LTL	Petrović et al. (1993)
	Chicken PM	Huber & Stadelman (1970)
ATPase activity	Beef SD	Wagner & Añón (1985)
	Chicken PM and BF	Khan & van den Berg (1967)
Surface hydrophobicity	Pork LTL	Zhang & Ertbjerg (2019)
Denaturation enthalpy and peak temperature on DSC	Pork LTL	Zhang & Ertbjerg (2019)
	Beef SD	Wagner & Añón (1985)
Water distribution on NMR	Pork LTL	Mortensen, Andersen, Engelsen, & Bertram (2006)
WHC of myofibrils	Pork LTL	Zhang & Ertbjerg (2019)
WHC of meat	Pork SM, SD, BF	Ku, Jeong, Park, Jeon, Kim, & Kim (2014)
	Beef LTL	Petrović et al. (1993)
Protein content in thaw drip	Chicken PM and BF	Khan & van den Berg (1967)
	Chicken breast	Khan (1966)
Near-infrared spectroscopy	Pork LTL	Xie, Sun, Zhu, & Pu (2016)

LTL *M. longissimus thoracis et lumborum*; SD *M. semitendinosus*; SM *M. semimembranous*; PM *M. pectoralis major*; BF *M. biceps femoris*

2.4.3. Other freezing-induced changes related to thaw loss

It is well demonstrated that the structural constraint forces e.g. exerted by cytoskeleton, Z-disks and cross-bridges within the muscle structure could prohibit the unlimited swelling occurring within the

myofibrils thus affecting WHC (Offer & Knight, 1988a; Puolanne and Halonen 2010). Kristensen & Purslow (2001) has proposed that degradation of cytoskeleton proteins due to postmortem proteolysis is causing improved WHC during aging as illustrated in Fig. 5. The cytoskeletal proteins are gradually degraded due to proteolysis with prolonged postmortem time, thus resulting in a loosened structure within the muscle fiber shown as Fig. 5C. The extracellular water would then flow back from outside to inside of the muscle fiber, thus potentially contributing to improved WHC in the later periods of aging.

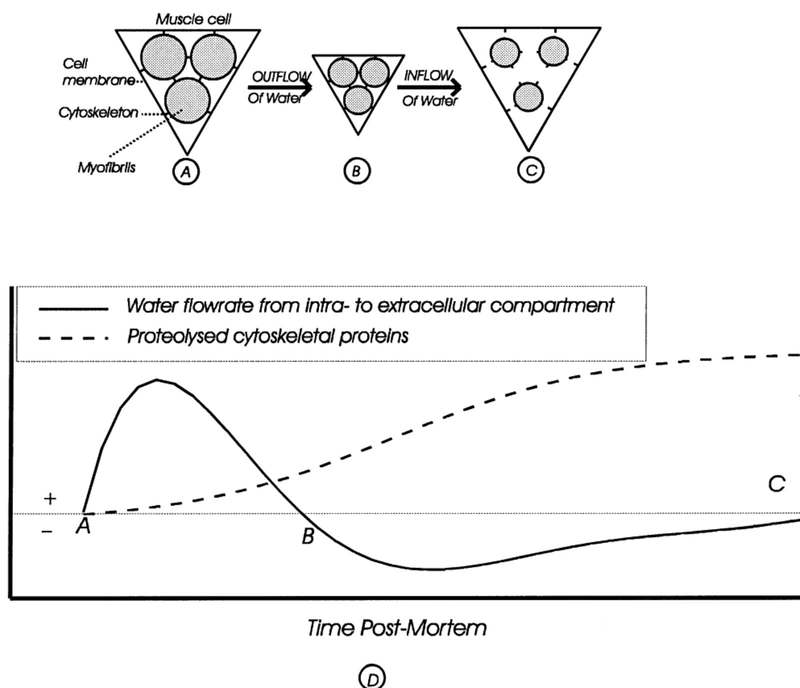

Fig. 5. Hypothesis to explain improved WHC in relation to the degradation of cytoskeleton proteins. (A) An illustrated pre-rigor muscle fiber in which three myofibrils connected to each other and to the cell membrane through the cytoskeleton. (B) Myofibrils shrink postmortem resulting in a shrinkage occurring in the whole muscle fiber, accompanied with a migration of water from intra- to extracellular space. (C) Proteolysis with prolonged postmortem time removes the constraint forces on the cell membrane thus causing an inflow of water from outside to inside of muscle fiber. (D) Relationship between water flow rate, postmortem time, and proteolysis (Kristensen & Purslow, 2001).

The changes in the myofibrillar volume have been proposed as the main causative factor in relation to WHC (Hamm, 1960; Offer & Knight, 1988a). The transverse elements e.g. Z-disks and intermediate

filaments that exist to maintain the myofibril structure could thus in turn prohibit the swelling or shrinking of myofibrils, consequently influencing WHC during aging. Recently, Zeng, Li, & Ertbjerg (2017) has proposed a hypothesis that the swelling inside of the myofibrils induced by desmin degradation and α-actinin release occurring in and around the Z-disks could potentially allow water to flow back from the area outside to inside of myofibrils thus contributing to improved WHC in the process of aging (Fig. 6).

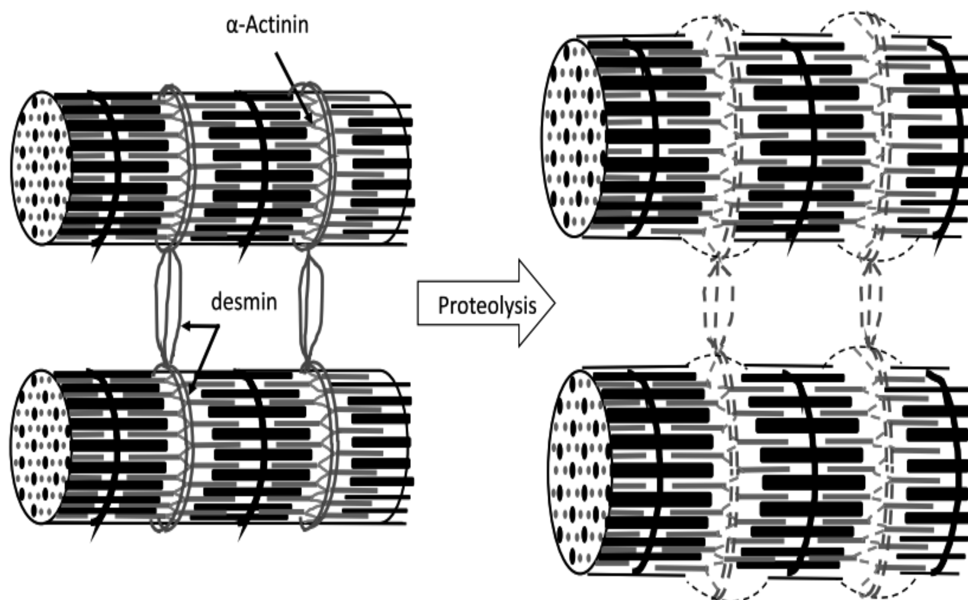

Fig. 6. Hypothesis illustrating the structural changes within the myofibrils resulting from proteolytic degradation of cytoskeletal proteins i.e. desmin and α-actinin (Zeng, Li, & Ertbjerg, 2017).

The calpains are generally believed to be the most important proteolytic enzymes within the meat tissue. Calpain-1 and calpain-2 are recognized as the two best-characterized isoforms within calpains, and their activity often depends on the concentration level of free Ca^{2+} in the sarcoplasm as well as the inhibitor calpastatin. It is well known that some cytoskeletal proteins e.g. desmin, filamin and vinculin are substrates for the calpains (Baron, Jacobsen & Purslow, 2004). Therefore, the enzyme activity of calpains is highly associated with the proteolytic degradation of the cytoskeleton consequently affecting WHC. Freezing and thawing have been shown to produce greater proteolysis as indicated by desmin degradation (Grayson et al., 2014) as well as increased fragmentation index (Aroeira et al.,

2016). Wheeler, Crouse, & Koohmaraie (1992) reported decreased extractable activity calpain-1 after freezing and thawing followed by aging for 6 day. As observed by Whipple & Koohmaraie (1992), calpastatin activity was significantly decreased following freezing and thawing. It was shown in a recent study (Colle et al., 2018) that freezing and thawing caused a distinct decrease by around 40% of calpain-2 activity in beef muscle. Cathepsins play a role in proteolysis (Wang et al., 2014; Zeng, Li, & Ertbjerg, 2017). Yamamoto, Samejima & Yasui (1977) has attributed the bands between 140 and 200 kDa which appeared in frozen-thawed hen muscle to the myofibrillar protein degradation induced by the release of lysosomal cathepsins into sarcoplasm due to the disruption of muscle fibers occurring in freezing-thawing. These observations thus potentially indicate a role of freezing-thawing in affecting activity of proteases and proteolysis, consequently affecting WHC of meat during the subsequent chilled storage. The proteasome is also an important proteolytic enzyme complex existing in the meat tissue, and some studies have indicated that the proteasome could contribute to proteolysis by hydrolyzing myofibrillar proteins and releasing α-actinin during cold storage (Houbak, Ertbjerg, & Therkildsen, 2008; Zeng et al., 2017). However, the effect of freezing and thawing on the proteasome activity is still lacking.

The formation of thaw loss is generally not avoided when meat is frozen and then thawed. Many studies conducted on freezing and thawing have reported decreased WHC of meat in terms of thaw loss, drip loss and cooking loss (Añón and Calvelo, 1980; Hamm, 1986; Leygonie et al., 2012a). The damage to muscle fibers resulting from ice crystallization and protein denaturation in freezing and thawing could potentially lead to migration of water from inside to outside of muscle fibers and consequently cause increased water loss upon thawing. The freezing rate has been well recognized as a contributor to the size and distribution of ice crystals formed within the frozen muscle. Some studies have over the years observed myofibrillar protein denaturation occurring in freezing, thawing and frozen storage. With regard to the interactions between myofibrillar proteins and water within the muscle fibers, the effect of the denaturation of myofibrillar proteins on WHC is thus to be expected. Nevertheless, the role and relative importance of protein denaturation in the generation of thaw loss is still not well understood.

3. Objectives

The aim of this thesis was to investigate freezing-induced protein denaturation occurring in the myofibrillar proteins in relation to the characteristic of WHC and to understand the role of myofibrillar protein denaturation in the generation of thaw loss. To achieve this, the frozen-then-chilled storage and the freezing rate corresponding to fast and slow freezing were applied to investigate the changes of proteolytic enzymes and protein denaturation occurring in freezing-thawing. The specific aims were:

- To evaluate the effect of frozen-then-chilled storage on proteolytic enzymes activity and WHC related to thaw loss and myofibrillar protein characteristics of porcine LTL muscle (Study I)

- To investigate the relationship between the freezing rate and the denaturation of protein in the myofibrillar and sarcoplasmic fractions and the influence of their relationship on the formation of thaw loss (Study II)

- To investigate the combined effect of low pH and high ionic strength on myofibrillar protein denaturation in relation to WHC in order to mimic conditions found in freezing-thawing, and to provide new insight on freezing-induced protein denaturation (Study III)

- To investigate the effect of the subsequent storage at -3 °C on myofibrillar protein characteristics in fast or slow frozen pork muscle in order to study protein denaturation potentially occurring in the industrial thawing-storage (Study IV)

4. Materials and methods

The materials and methods applied in the current thesis are briefly described in this section. More details can be seen in the attached publications (I, II, III & IV).

4.1. Sample processing

Muscle

Porcine LTL muscles used for the whole thesis were collected from HKScan Ltd slaughterhouse (Forssa, Finland), and they were transported cooled to the meat laboratory at University of Helsinki at 10 h postmortem. The connective tissue and external fat were trimmed out of each muscle. It should be noticed that frozen-thawed meat was processed at a longer postmortem time compared to fresh when considering the time of the freeze storage in the current thesis. The length of freeze storage was kept as short as possible in order to diminish the effect of protein and lipid oxidation occurring during freeze storage.

Sampling

Eight LTL muscles obtained from eight different pigs in Study I were each divided into ten pieces at 12 h postmortem, and each piece of approximately 200 g was vacuum packaged. A small proportion (around 30 g) from each muscle was then collected and frozen in liquid nitrogen, being used as 12 h postmortem reference samples for calpain analysis. The ten pieces were assigned to two treatments (non-frozen or frozen-thawed) with five chilled storage periods (1, 2, 4, 6 and 9 days) at 2 ± 1 °C. Meat pieces were frozen at -20 °C and thawed at 2 °C overnight which were defined as the frozen-thawed samples.

Samples from six porcine LTL muscles at 24 h postmortem were used in Study II & III. In Study II 12 pork cuts (around 9 × 6 × 2 cm along the muscle length) cut from each muscle were individually vacuum packaged and were then subjected to one of three treatments: 1) non-frozen control, 2) fast freezing at -80 °C and 3) slow freezing at -18 °C. The internal temperature of each pork cut was monitored during freezing. As suggested by Anõn and Calvelo (1980) and Bevilacqua et al. (1979), the characteristic freezing time (t_c) were categorized as being below 15 min for fast freezing and longer than 23 min for slow freezing. The observed t_c value in the current thesis were respectively 12 min and 174 min for fast and slow freezing meat, and thus could be recognized as categories of fast and slow freezing (Grujić et al., 1993; Kim et al., 2018). All frozen samples were stored at -80 °C until further

analysis. Thawing was operated at 2 °C. Additionally, exposure of the isolated myofibrils to combinations of different pH (from 5.5 to 5.0) and ionic strength (from 0.15 to 1.5 M) was operated in Study II, and the myofibril pellets isolated from five fast frozen-thawed LTL muscles were resuspended in MES buffer (50 mM MES, 2 mM $MgCl_2$, and 2 mM EGTA) with different KCl concentrations and pH. This procedure was repeated twice more following centrifugation. To restore the pH 5.5 and low ionic strength, the myofibril pellets were washed with cold buffer (75 mM KCl, 100 mM MES, 2 mM $MgCl_2$, 2 mM EGTA, pH 5.5) before further analysis.

In Study III three batches of minced meat were used, and each batch including two porcine LTL muscles was then divided into two parts. One part was used for making minced meat patties (9 cm in diameter × 1.5 cm in thickness) by a round burger mold. These patties were individually weighed and vacuum packaged, and were then subjected to one or two freezing and 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). Another part was mixed with different amounts of KCl and lactic acid to achieve the exposure of minced meat to lower pH at high ionic strength including six groups: 1) fresh and 2) freezing control both at pH 5.5 without KCl added, and fresh minced meat mixed with 2 M KCl and exposed to 3) pH 5.5, 4) pH 5.4, 5) pH 5.3 and 6) pH 5.2 achieving by adding different concentrations (0.1 M, 0.2 M or 0.3 M) of lactic acid. The KCl amount was adjusted to 2 M according to the measured chloride content in the supernatant after centrifuging minced meat samples. The chloride content was determined by a Corning 926 Chloride Analyzer (Corning Ltd, Halstead, GB). The freezing control used fresh samples which were frozen at -18 °C and stored for 5 days and then thawed at 2 °C. Myofibrils were prepared as described above and restored at pH 5.5 and low ionic strength by washing in a MES buffer.

In Study IV five porcine LTL muscles at 48 h postmortem were each divided into 18 pork cuts, and each cut (around 9 × 6 × 1.5 cm) was vacuum packaged and then subjected to two freezing treatments (fast and slow freezing) with four subsequent storage periods at -3 °C (0, 1, 3 and 7 days). The freezing procedure was operated as described in Study II, and the final thawing process was done after samples were kept at -3 °C for different number of days.

4.2. Physical analysis

The determination of pH

Postmortem pH for all meat samples and the pH for the exposure procedure in Study III were both determined by directly inserting a pH electrode (Mettler-Toledo Inlab 427) into meat tissue. Meat homogenate was used for the pH measurement in Study IV.

Purge loss and cooking loss

Fresh meat samples were weighed to record the initial weight before freezing. Following treatments (freezing-thawing or chilled storage), meat samples were blotted dry with filter paper and the weights were then recorded. Purge loss was calculated by comparing the weight difference of samples before and after treatments (expressed as percentage loss), and the thaw loss was thus included for freezing-thawing samples. Cooking loss (Study I) was measured as the percentage loss of the weight of pork cuts before and after cooking. The sum of the purge and cooking loss was defined as the total loss (Study I).

WHC of myofibrils

WHC of myofibrils was determined based on a centrifugation method (Bao, Boeren, and Ertbjerg, 2018). Isolated myofibril pellets were resuspended in a MES buffer (pH 5.5) followed by centrifugation at 2,400×g for 10 min, and the supernatant was then discarded. The weight difference of the pellet before and after drying in an oven at 100 °C was used for calculating the changes of the water amount within the pellet. WHC was thus defined as the amount of water held per gram of myofibrillar protein within the pellet.

Allo-Kramer shear force

Allo-Kramer shear force (Study I) was measured according to the method described by Liu, Ruusunen, Puolanne, & Ertbjerg (2014). The pork cuts were cooked at 72 °C for 60 min followed by cooling in cold water for 30 min, were then divided into small slices of 20 × 20 × 6 mm (fiber along the 20 mm direction) and the Allo-Kramer shear cell was used for the measurement of shear force.

Free Ca^{2+} concentration

Free Ca^{2+} concentration (Study I) was determined based on a method of Ca^{2+} standard addition using a Ca^{2+} ion selective electrode (Pomponio and Ertbjerg, 2012). The meat sarcoplasm was collected by centrifuging chopped samples at 20,000×g for 30 min at 4 °C, and 4 mL aliquots were mixed with 80

µL of KCl (4 M) for internal adjustment when using the Ca^{2+} ion selective electrode. A calibration curve was established to describe the relationship between the Ca^{2+} concentration and millivolts.

Particle size

The particle size distribution of the isolated myofibril suspensions (Study II & III) was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK). For each suspension the analysis was repeated five times using distilled water as dispersant. The refractive index and the absorption coefficient were set to 1.46 and 0.01, respectively. D(v,0.1) and D(v,0.9) are used to describe the size of the particle for which 10% and 90% of the sample is below the corresponding size, respectively. D(3,2) and D(4,3) represent the mean diameter in surface and volume, respectively.

Intrinsic fluorescence measurement

The meat drip was collected at 72 h postmortem in Study II, and the intrinsic fluorescence emission spectra were determined on a LS 55 Luminescence Spectrometer (PerkinElmer Inc., Waltham, MA, USA) according to the method described by Estévez, Kylli, Puolanne, Kivikari, and Heinonen (2008) with modifications. The protein content in the meat drip was adjusted to 0.015 mg/mL before measurement. The excitation wavelength was set at 295 nm and the emission spectra were recorded from 300 to 400 nm scanned at 240 nm/min. The slit width was set as 7 nm.

4.3. Biochemical analysis

Calpain activity

Calpain activity in Study I was measured by casein zymography as described by Pomponio and Ertbjerg (2012). Frozen samples were homogenized in cold buffer (100 mM Tris-HCl buffer, 5 mM EDTA, and 10 mM monothioglycerol, pH 8.3). The supernatant was collected following centrifugation and then mixed with glycerol achieving a final concentration of 30% in order to protect enzyme activity during freezing and thawing. The procedures for making casein gels and running the electrophoresis as well as the subsequent incubation were described in 2.3, Study I. Coomassie Brilliant Blue G-250 was used for staining gels. The native calpain-1 and -2 activities at 12 h postmortem were taken as a reference standard (100%), and the native and autolyzed calpain activities were then calculated as a relative intensity percentage to the reference standard.

Proteasome activity measurement

The chymotrypsin-like activity of the proteasome in the supernatant (from 2.2, Study II) was measured by using carbobenzoxy-Gly-Gly-Leu-7-amido-4-methylcoumarin (Z-GGL-AMC) (Sigma, Saint Louis, MO) as a substrate. The incubation was operated for 6 min at 30 °C in an activity buffer (20 mM Tris-HCl, 5 mM EDTA, and 10 mM monothioglycerol, pH 7.7). The AMC was then released from the substrate. Spectrofluorometer Infinite M200 scalable microplate reader (Tecan, Mannedorf, Germany) was used to detect the changes of fluorescence every 2 min for a total of 30 min. The excitation wavelength was set to 360 nm and the emission wavelength to 450 nm. The activity of proteasome was expressed in μM / (min × g meat).

Surface hydrophobicity of myofibrils

Surface hydrophobicity of the isolated myofibrils (Study II, III & IV) was measured following the method described by Liu et al. (2016). The protein content in the myofibril suspension was measured according to the method of DC Protein Assay Kit. The myofibril suspension (adjusted 2 mg/mL) was then incubated with bromophenol blue (BPB) (1 mg/mL) for 10 min at room temperature and the supernatant was collected after centrifugation. MES buffer without myofibrils was added to the same volume of BPB as the control. The absorbance was measured at 595 nm. The surface hydrophobicity was expressed as the amount of BPB bound per mg myofibrillar protein.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was operated in Study II by using a DSC 823^{e} (Mettler Toledo AG, Greifensee, Switzerland) according to the method as described by Voutila, Perero, Ruusunen, Jouppila, and Puolanne (2009). The weight of the empty sample pan was recorded and meat samples of around 30 mg (or purge drip of 30 μL) were then weighed in the aluminum sample pan, and the pans were then hermetically sealed before measuring. The heating temperature was set from 25 to 95 °C at a rate of 5 $°C \times min^{-1}$ and the heat flow was recorded in the program. An empty sample pan was used as a reference. Following DSC analysis sample pans were pierced and then dried at 105 °C overnight. The corresponding temperature for each peak (T_{peak}) and denaturation enthalpy (ΔH) were used here to describe the characteristics of protein denaturation. The dry matter weight was calculated, and ΔH values were expressed as joules per gram dry matter.

SDS-PAGE and protein identification

Myofibrillar protein suspension was prepared for SDS-PAGE analysis in Study III & IV. Protein content was measured according to the method of DC Protein Assay Kit. Protein suspension was then diluted to around 2.3 mg/mL followed by mixing with NuPAGE^{TM} LDS Sample Buffer (4 ×) and NuPAGE^{TM} Sample Reducing Agent (10 ×) (Invitrogen, Carlsbad, CA). The mixture was incubated at 70 °C for 10 min, and then loaded onto NuPAGE^{TM} Novex 3-8% Tris-Acetate gels. The electrophoresis was performed at 150 V for around 50 min. Coomassie Brilliant Blue R-250 was used for staining gels. The band of interest in gels was excised, and the identification of the protein was followed the method as described by Vaarala et al. (2014) based on mass spectrometry analysis.

4.4. Statistical analysis

Data analysis was carried out using general linear model in the IBM SPSS Statistics 24 software. Treatment was arranged as a fixed factor, and animal number (Study I, II & IV) and batch number (Study III) as random factor. The significant differences between mean values were evaluated by the Bonferroni and Tukey HSD (honest significant difference) tests with a level at $P < 0.05$.

5. Summary of results

5.1. Effect of freezing-thawing on meat quality regarding changes of proteolytic enzymes and WHC of meat

Changes of proteolytic enzymes

Freezing and thawing significantly affected the concentration of free Ca^{2+} and the proteolytic enzymes activities of calpains and the proteasome during the subsequent chilled storage (Fig. 1, 2 and 3, Table 1, Study I). The initial free Ca^{2+} concentration in the non-frozen porcine LTL muscle was 140 μM on Day 1 (24 h postmortem) and significantly increased to 400 μM after 9 days of chilled storage. In comparison, a distinct increase ($P < 0.05$) of free Ca^{2+} concentration up to 420 μM was observed in the frozen-thawed group on Day 1, and gradually reached to 510 μM on Day 9. Free Ca^{2+} is an activator of calpains. The native calpain-1 activity decreased by around 25% in the frozen-thawed group compared to that of non-frozen group on Day 1, and a disappearance of the calpain-1 activity was found on Day 2 for the frozen-thawed group and on Day 4 for the non-frozen samples. Moreover, the native calpain-2 and autolyzed calpain-2 activity were significantly affected by the freezing-thawing with increased chilled storage. The native calpain-2 in the non-frozen samples reduced its activity by around 20% from Day 1 to Day 9, but up to 55% was lost in the frozen-thawed samples after 9 days of chilled storage. The autolyzed calpain-2 activity of non-frozen samples was observed on Day 4 and of frozen-thawed samples on Day 2. In addition, around 25% of autolyzed calpain-2 activity was found in the frozen-thawed samples on Day 9. The chymotrypsin-like activity of the proteasome was significantly reduced by around 40% on Day 1 due to freezing and thawing, and lower activity was observed in the frozen-thawed group in comparison with the non-frozen group stored at the same period.

Changes in WHC

In this thesis, purge loss of non-frozen pork cuts was measured during the period of the chilled storage, and for frozen-thawed samples, purge loss was defined as the sum of water losses during thawing and the subsequent chilled storage. A greater purge loss ($P < 0.05$) was generally found in the frozen-thawed samples when compared to the non-frozen samples (Fig. 4B, Study I; Fig. 2A, Study II; Fig. 1A, Study III). Additionally, the effect of the freezing rate was observed in which purge loss was on average 30% greater in slow compared to fast freezing (Fig. 2A, Study II; Fig. 1A, Study III; Fig. 1,

Study IV). However, with prolonged storage at -3 °C until Day 7, no difference ($P > 0.05$) in purge loss was observed between fast and slow freezing (Fig. 1, Study IV). When compared to the non-frozen samples, the frozen-thawed samples showed a higher cooking loss ($P < 0.05$) on Day 1, and thereafter the cooking loss remained relatively stable until the end of storage (Table 2, Study I). The total water loss was expressed as the sum of the purge and cooking loss (Table 2, Study I), and a greater total exudate loss ($P < 0.05$) was observed in the frozen-thawed samples throughout the chilled storage, compared to the non-frozen samples.

5.2. Effect of freezing and freezing rate on protein characteristics

Myofibrillar proteins

Myofibrillar proteins representing 60% to 70% of the total meat proteins, thus play an important role in controlling the WHC in the muscle structure as the major part of muscle water is held within the myofibrils. WHC of myofibrils measured here was thus used to detect the changes of the amount of water held by the isolated myofibrils potentially attributed to the physicochemical changes within myofibrillar proteins. Freezing and thawing affected ($P < 0.05$) the WHC of myofibrils, and the frozen-thawed samples showed lower values of WHC when compared to the non-frozen samples (Fig. 4A, Study I; Fig. 2B, Study II; Fig. 1B, Study III). An effect ($P < 0.05$) of an additional freezing and thawing cycle was observed in which myofibrils extracted from two freezing-thawing cycles showed the lowest WHC when compared to that from non-frozen or one cycle (Fig. 1B, Study III). In frozen-thawed samples, the freezing rate produced a distinct effect on WHC of myofibrils (Fig. 2B, Study II; Fig. 1B, Study III) and that fast freezing showed around 4% greater values when compared to slow freezing. The effect of the freezing rate on WHC of myofibrils was also observed when deep frozen pork samples were kept at storage of -3 °C within 3 days before final thawing at 2 °C, but this difference was not significant after 7 days (Fig. 4, Study IV).

Surface hydrophobicity has previously been used to indicate protein denaturation, and a greater value generally indicates a more pronounced protein denaturation in the myofibrils. Freezing rate in this thesis was shown to significantly affect the surface hydrophobicity in the isolated myofibrils (Fig. 2C, Study II; Fig. 1C, Study III; Fig. 3, Study IV), and the order of the values between different groups was non-frozen $<$ fast freezing $<$ slow freezing.

Major endothermic peak transitions observed from the DSC thermogram have often been used as indicators for protein denaturation. Three endothermic peaks were found in DSC curves for pork LTL muscle (Table 1 and Supplemental material 2, Study II). The first and the third peak have previously been ascribed to myosin and actin, respectively. The treatment of freezing and thawing affected the peak transition temperature, and non-frozen samples showed slightly higher ($P < 0.05$) temperatures for the first and third peak when compared to the two frozen-thawed samples. With regard to the denaturation enthalpy for the first peak, the non-frozen samples showed a higher ($P < 0.01$) value of 1.54 J×g^{-1} when compared to the values being 1.47 and 1.42 J×g^{-1} in the fast and slow freezing samples, respectively. However, an opposite trend was observed in the third peak in which the denaturation enthalpy was lower ($P < 0.01$) in non-frozen samples compared to that in two frozen-thawed groups. Additionally, no difference was noticed either in the peak transition temperature or the denaturation enthalpy, between fast and slow freezing groups.

The freezing-thawing process also showed an effect on myofibrillar protein degradation or aggregation. Protein profiles in the myofibrillar fraction were studied by SDS-PAGE, and a distinct protein band at a position around 160 kDa was observed only in the myofibrils isolated from frozen-thawed meat as compared to the fresh (Fig. 3, Study III; Fig. 5, Study IV). Additionally, the intensity of this protein band increased in response to increased freezing-thawing cycles (Fig. 3, Study III) or prolonged subzero temperature storage at -3 °C (Fig. 5, Study IV). This protein band was identified as a myosin fragment originating from myosin-4 (MYH4) with a native molecular weight of 224 kDa by mass spectroscopy (Study III). Particle size distribution was used as an indicator to assess the structural changes of myofibrillar proteins (Lametsch, Knudsen, Ertbjerg, Oksbjerg, & Therkildsen, 2007). As shown in Fig. 2 (Study III), slow freezing and two cycles of slow freezing both significantly increased values of the investigated parameters of particle size (D(3,2) and D(v,0.1)) when compared to the fresh samples, potentially indicating a formation of larger particles in the myofibrils in relation to slow freezing and thawing. However, no effect was found due to fast freezing and two cycles of fast freezing.

Sarcoplasmic proteins

The denaturation of sarcoplasmic proteins occurring during freezing and thawing was evaluated as changes in the DSC profiles and the intrinsic fluorescence spectra (Table 1 and Fig. 3, Study II). Three endothermal peaks were found in DSC curves for meat samples, and the middle one has been ascribed

to sarcoplasmic proteins and collagen denaturation. Lower peak transition temperature and denaturation enthalpy ($P < 0.05$) were observed for the middle peak in the slow freezing group as compared to the non-frozen samples. The purge drip collected from the fresh and two frozen-thawed samples was used to investigate thermal stability of sarcoplasmic proteins in detail and no difference of the protein content in drip was found between fresh and froze-thawed samples. One endothermal peak was shown in the DSC profile of drip indicating a denaturation occurring in the sarcoplasmic proteins. Compared to fresh samples, purge drip collected from frozen-thawed groups showed reduced ($P < 0.05$) denaturation enthalpy, but no effect of freezing rate was found. Freezing and thawing showed an effect on the intrinsic fluorescence spectra. Purge drip collected from fresh samples showed a distinct difference of the fluorescence spectra in comparison with that from frozen-thawed samples, whereas similar fluorescence spectra were observed between fast and slow frozen samples. The maximum fluorescence emission wavelength did not show any shifts following the freezing-thawing process. However, increased fluorescence intensity was observed in the two frozen-thawed samples when compared to the fresh samples.

5.3. Effect of the combination of low pH and high ionic strength on myofibrillar protein characteristics

Based on the assumption that more than 90% of muscle water would be frozen below -20 °C, it is thus to be expected that a > 10-fold increase in proton concentration and ionic strength would occur in the unfrozen water during freezing of meat consequently contributing to protein denaturation. Therefore, this thesis investigated the combined effect of low pH and high ionic strength on myofibrillar protein attributes occurring in isolated myofibrils (Table 2, Study II) and in minced meat (Fig. 4 and 5, Study III) in order to study changes of myofibrillar proteins caused by freezing and thawing.

Treatment of isolated myofibrils

Isolated myofibrils were exposed to combinations of decreased pH (from 5.5 to 5.0) and increased ionic strength (KCl concentration from 0.15 to 1.5 M), and their combined effect on myofibrillar protein attributes were assessed by WHC, surface hydrophobicity and particle size of myofibrils (Table 2, Study II). The exposure to pH 5.5 at 0.15 M of KCl concentration showed the highest value of WHC, and the lowest value for surface hydrophobicity and particle size parameters. Declined pH combined

with increased KCl concentration resulted in reduced WHC and increased values of surface hydrophobicity and particle size when compared to the combination of pH 5.5 and 0.15 M KCl, and more interestingly, these distinct changes were observed onwards from around pH 5.2 and 1 M of KCl.

Treatment in minced meat

Fresh minced pork LTL muscle was exposed to lower pHs (from 5.5 to 5.2) combined with high ionic strength (corresponding to 2 M KCl) in order to study conditions in freezing, and the effects of the exposure on myofibrillar protein characteristics were investigated by observing the changes of WHC, surface hydrophobicity value and SDS-PAGE profile of myofibrils. The pH and ionic strength of the isolated myofibrils used here for analysis were restored to the original levels before measurements. With regard to WHC of myofibrils, freezing-thawing resulted in a distinct decrease ($P < 0.05$) by around 18% when compared to the non-exposed fresh control, whereas an increase ($P < 0.05$) by around 9% was observed following the exposure of minced meat to high ionic strength corresponding to 2 M KCl at pH 5.5. Therefore, exposure to only high salt did not reproduce the structural alterations within the myofibrils in freezing-thawing. However, when fresh minced meat was exposed to lower pH values (pH 5.4, 5.3 and 5.2) combined with high salt (2 M KCl), a progressive decline ($P < 0.05$) of WHC values was observed and the lowest value occurred at pH 5.2 with high salt. The combination of pH 5.3 and high ionic strength (corresponding to 2 M KCl) showed a similar value of WHC in the isolated and washed myofibrils compared to that of myofibrils isolated from frozen-thawed meat.

Surface hydrophobicity was observed to increase ($P < 0.05$) in the frozen-thawed meat when compared to the non-exposed fresh control. Greater values for surface hydrophobicity were observed in the exposure to high ionic strength combined with decreased pH and the greatest value was shown at pH 5.2 with high salt. SDS-PAGE was used to investigate protein profiles in the myofibrillar fraction, and freezing resulted in the appearance of a band migrating at a position around 160 kDa which was at the same position with the myosin-4 fragment found following the freezing and thawing cycles (Fig. 3, Study III). The exposure of fresh minced meat to high salt and a lower pH also supported the appearance of the same band, and additionally the intensity of this band was observed to increase in response to exposure at lower pHs.

6. General discussion

6.1. Effect of freezing-thawing on meat quality in relation to proteolytic enzyme activity and water loss upon thawing

Changes of proteolytic enzyme activity

The calpain system has been well recognized as an important contributor to postmortem proteolysis and the resultant tenderization of meat (Huff-Lonergan & Lonergan, 2005; Koohmaraie & Geesink, 2006). The free Ca^{2+} is an activator for calpains and the concentrations required to activate calpains are in the range of 3-50 μM for calpain-1 and 400-800 μM for calpain-2 (Goll, Thompson, Li, Wei, & Cong, 2003). Early postmortem the free Ca^{2+} concentration has been reported to be greater than 100 μM (Geesink, Taylor, Bekhit, and Bickerstaffe, 2001), thus being sufficient for the activation of calpain-1. In the current study, the freezing-thawing process caused a three-fold increase in free Ca^{2+} resulting in more than around 400 μM of free Ca^{2+} in the sarcoplasm observed on Day 1 (Fig. 1, Study I), and this value was similar to the free Ca^{2+} concentration after 9 days of fresh storage. This increase of free Ca^{2+} could then accelerate the activation and autolysis of calpain system, which would reduce the stability of calpain system consequently leading to loss of activity (Goll, et al., 2003). As observed in Study I, the extractable activity of calpain-1 was significantly declined in the frozen-thawed samples and it was not detectable on Day 2 when compared to the disappearance of calpain-1 activity occurring on Day 4 in the non-frozen samples (Fig. 2, Study I). A role of calpain-2 has been suggested in proteolysis of pork (Pomponio, Lametsch, Karlsson, Costa, Grossi, & Ertbjerg, 2008) and of beef in later postmortem (Colle & Doumit, 2017). The calpain-2 activity in the non-frozen samples was observed to decline by around 20% after 9 days of chilled storage accompanied with the increase of autolyzed calpain-2 activity (Fig. 2, Study I) which is likely attributable to the increase of free Ca^{2+} concentration to around 400 μM observed in the sarcoplasm of non-frozen pork after prolonged storage (Fig. 1, Study I). The freezing-thawing treatment significantly accelerated the decline of the native calpain-2 activity during the subsequent chilled storage when compared to the non-frozen samples (Fig. 2, Study I), but this effect seemed not to be obvious within 2 days of storage indicating that the observed increase in the free Ca^{2+} concentration in the frozen-thawed samples did not cause an instant activation of calpain-2 in the early storage. This delay might be related to the binding of free Ca^{2+} in the sarcoplasm to EF-hand structures or other Ca^{2+}-binding sites within calpain-2 molecules since some of these sites show low affinity for free Ca^{2+} and also more free Ca^{2+} is required to bind calpain-

2 for the activation (Goll et al., 2003). As observed by Colle et al. (2018), a decline by around 40% of calpain-2 activity was found in the frozen-thawed beef muscle with the subsequent aging, and they attributed it to the reduced requirements of free Ca^{2+} concentration for calpain-2 activation induced by the interaction of phospholipids with calpain-2 possibly occurring during freezing and thawing.

The proteasome has in some studies been suggested to involve proteolysis of some structural proteins (Houbak et al., 2008; Robert, Briand, Taylor, and Briand, 1999) which would consequently contribute to better WHC during chilled storage (Zeng et al., 2017). The chymotrypsin-like proteasome activity in the non-frozen samples was observed to decrease ($P < 0.05$) with increased chilled storage (Fig. 3, Study I), potentially indicating a negative effect of the proteasome in the meat tenderization during aging. As there are no literature data available about the effect of the freezing-thawing process on proteasome activity, Study I is the first study to show the loss of the proteasome activity in the frozen-thawed samples which could possibly cause reduced proteasome-related proteolysis during the subsequent chilled storage.

Changes in WHC

The freezing-thawing process was generally observed in the current thesis to cause a decrease in the WHC of meat shown as increased water loss upon thawing and greater cooking loss as well as more specifically, reduced WHC of myofibrils. In agreement, the majority of the research focused on freezing and thawing have documented a decrease of WHC in the frozen-thawed meat (Aroeira et al., 2016; Kim & Kim, 2016; Kim et al., 2018; Vieira, Diaz, Martínez, & García-Cachán, 2009). For example, when compared with the amounts of drip averaging 6.2% from non-frozen muscle, frozen-thawed pork has by Penny (1975) been found to achieve a further increase by around 7% units of thaw exudate loss. Multiple freezing and thawing cycles generally cause more water to be released. Cheng et al. (2019) observed increased thaw loss and cooking loss as well as a linear decrease in WHC when beef muslce was subjected to freeze-thaw cycles. Also, Ali et al. (2015) found decreased WHC of chicken breast muscle with increased freeze-thaw cycles, and they attributed it to the migration of water to the extracellular area during freezing and thawing based on the observation of a reduced relaxation time of the immobilized water in NMR measurement.

The rate of freezing is known to influence the amount of earlier thaw loss (Hamm, 1986), and a larger amount is generally observed in slow than fast freezing (Kim et al., 2018; Petrović et al., 1993; Ngapo et al., 1999). The current thesis observed a substantial increase by around 30% of purge loss when pork muscle was frozen at the slow rate as compared with fast freezing rate (Fig. 2A, Study II; Fig. 1A, Study III; Fig. 1, Study IV). Ice crystal formation is often believed to be the main driving force for the generation of thaw loss (Leygonie et al., 2012a) and the rate of freezing is a contributing factor in the size and distribution of ice crystals formed during freezing (Bevilacqua et al., 1979; Bevilacqua & Zaritzky, 1980). Freezing at fast rate generally produces small ice crystals located uniformly within and between muscle fibers, while at slow rate, more intracellular water will be migrated osmotically to outside of the fibers to form large extracellular ice crystals thus causing cell dehydration. The mechanical disruption of muscle fibers caused by ice crystals formed in the intra- and extracellular spaces in relation to the freezing rate would potentially affect the reabsorption of water by muscle fibers upon thawing (Hamm, 1986). In the current study, the denaturation of myofibrillar proteins occurring during freezing and thawing as observed by decreased WHC of myofibrils, increased surface hydrophobicity, and reduced denaturation enthalpy and peak temperature is proposed to be the reason of the increased water loss upon thawing, which may indicate a role of protein denaturation in the formation of thaw loss.

Shear force

Freezing and thawing have been reported in beef muscle by Grayson et al. (2014) to decrease shear force or by Colle et al. (2018) not to affect shear force or sensory tenderness. In pork Kim et al. (2018) found reduced shear force value in frozen-thawed samples during the subsequent aging. In Study I, the freezing-thawing treatment did not produce an effect on the shear force of pork muscle but decreased values of shear force were observed with increased following chilled storage (Table 2, Study I). The accelerated activation of calpains occurring in frozen-thawed meat could induce degradation of structural proteins and hence meat tenderization (Huff-Lonergan & Lonergan, 2005; Pomponio & Ertbjerg, 2012). However, the reduced proteasome activity following freezing and thawing may suggest decreased proteolysis and thus negatively affect meat tenderness (Houbak et al., 2008; Thomas, Gondoza, Hoffman, Oosthuizen, and Naudé, 2004). Shear force has by Girard, Bruce, Basarab, Larsen, & Aalhus (2012) been reported to correlate to purge loss and cooking loss in beef muscle. It is to be

expected that there are more filaments in a cut surface after more myowater gets out. Thus, the observed substantial water loss upon thawing in the current thesis could possibly cause a more compact structure of muscle fibers, resultantly contributing to meat toughness (Leygonie et al., 2012a).

6.2. The importance of decreased pH at high ionic strength in the denaturation of myofibrillar proteins

In freezing below -20 °C more than 90% of the muscle water is frozen which could theoretically result in a higher than a 10-fold increase in solute concentration in the unfrozen water outside of the ice crystals. Protons would be expected to accumulate with concentrating solutes theoretically causing a one-unit decrease in pH in the remaining unfrozen phase. The increased buffering capacity of meat proteins observed at pH values lower than 5.5 (Puolanne and Kivikari, 2000) would, however, possibly absorb part of the excess protons. Temporarily, in the freezing-thawing process, it is to be expected that the water-accessible surface areas of myofibrillar proteins, e.g. myosin and actin filaments, would be exposed to a lower pH and higher ionic strength occurring in the unfrozen water phase. The contribution of pH and ionic strength to the protein unfolding is significant (Kristinsson & Hultin, 2003; Lin & Park, 1998), and therefore, an effect on the WHC within myofibrils is to be expected. Exposure to increased NaCl concentration (up to 2 M) introduced swelling of the isolated myofibrils (Knight & Parsons, 1988) and better WHC of meat (Hamm, 1986), whereas the combinations of low pH (from 5.4 to 4.6) and higher ionic strength (up to 1.6 M) have been found to cause a loss of WHC in meat (Puolanne and Peltonen, 2013). Also the exposure of the isolated myofibrils to a lower pH (around 5.2) combined with a higher KCl concentration (around 1 M) was observed in Study II to cause myofibrillar protein denaturation as evidenced by decreased WHC of myofibrils and increased surface hydrophobicity, which showed comparable properties to that observed in myofibrils isolated from frozen-thawed meat. However, salting before freezing has by Jiang et al. (2019a) been observed to improve WHC of tuna meat after multiple freezing and thawing cycles, and therefore, it can be questioned if increased ionic strength in the unfrozen water can be directly linked to an increased water loss upon thawing. The involvement of decreased pH combined with high ionic strength is to be expected to explain the loss of the amount of water held within myofibrils in relation to the denaturation of myofibrillar proteins in freezing-thawing.

A model is proposed in Study III to provide new insights into the mechanism behind of the freezing-induced protein denaturation mainly based on the observed effect of varied pH combined with high ionic strength on protein structure and the related alterations occurring within myofibrils (Fig. 7). Myosin filaments are net negatively charged in postmortem meat at pH 5.5 and low ionic strength (Fig. 7A). Protein denaturation occurring during freezing has conventionally been attributed to increased ionic strength in the unfrozen water phase as reviewed by Shenouda (1980) and Xiong (1997). The contribution of a potential decline of pH in the remaining water resulting from concentrating protons to the protein denaturation is to be expected. Therefore, Study III exposed fresh meat to combinations of pH and high salt concentration (2 M KCl) in order to simulate the hypothetical effect of high ionic strength combined with or without lower pH on myosin in freezing. The 2 M KCl used in Study III was corresponded to a high ionic strength that is close to 10 times larger than the original ionic strength of 0.19 in fresh meat (Offer & Knight, 1988) to stimulate a 10-fold increase in solute concentration in the unfrozen water that might occur in freezing at -20 °C (Calvelo, 1981; Finn, 1932). A pH of 5.5 and low ionic strength were then restored in the isolated myofibrils before the measurements, considering that the higher ionic strength and lower pH prevailing in freezing will return to the original with the dilution of the unfrozen water as the ice crystals melts during thawing.

The exposure to 2 M KCl only in our model (Fig. 7B) was to simulate the effect of high ionic strength (without pH decline) on myosin in freezing-thawing. As reviewed by Puolanne & Halonen (2010), the myosin rod has many positively charged amino acids that could attract chloride ions, and many negatively charged side chains that bind potassium ions. Additionally, chloride ions could also be absorbed to the hydrophobic core with a hollow structure within the myosin filaments. Therefore, the added KCl is suggested to increase the negative charges on the myofilaments, increasing the electrostatic repulsion within the myosin filaments thus introducing swelling of myosin (Hamm, 1986; Offer & Knight, 1988) which would consequently allow an increase of the cross-sectional area of sarcomeres (Fig. 7B). In agreement, the addition of salt has been observed by Chantarasuwan et al. (2011) to increase negative charges on the actomyosin surface and by Knight & Parsons (1988) to cause swelling of the isolated myofibrils. These alterations occurring within the myosin structure could thus provide an explanation for the observed greater WHC of myofibrils following the exposure to high ionic strength (Fig. 4A, Study III). However, the absorption of chloride ions to the hydrophobic core of myosin filaments would rupture the inner structure and cause the exposure of the inner

hydrophobic groups to water (Puolanned and Halonen, 2010), thus promoting the recognition of bromophenol blue and the hydrophobic sites on protein surface (Chelh et al., 2006), and consequently, increased surface hydrophobicity. Exposure to high salt was shown to increase WHC of myofibrils and decrease surface hydrophobicity when compared to the non-exposed control (Fig. 4, Study III), though the high salt (2 M KCl) used for the exposure was washed out of the isolated myofibrils before the measurements. It is thus to be expected that there were irreversible structural modifications occurring in the myofibrillar protein network, i.e. swelling of myosin filaments, induced by high salt during the exposure. Therefore, exposure to high ionic strength combined with a constant pH of 5.5 did not reproduce the decreased WHC of myofibrils as observed in frozen-thawed meat. The exposure to pH 5.3 and 2 M KCl in the model (Fig. 7C) was to mimic myosin changes in freezing-thawing hypothetically resulted from the effect of high ionic strength combined with a decreased pH. The addition of KCl would increase the negative charges on myofilaments thus causing an enlarged cross-sectional area. However, the involvement of a lower pH towards the isoelectric point reduces the net negative charges on myofilaments, thus potentially leading to a transverse shrinkage within sarcomeres and consequently also that of the fibers. The most likely scenario would then be that the effect on net charges caused by a pH decline could more than counterbalance the effect of high ionic strength, and therefore reduced negative charges in myofilaments and the resultant transverse shrinkage are to be expected in the sarcomere (Fig. 7C). Additionally, the combination of pH 5.3 and 2M KCl would induce more pronounced protein unfolding and consequently denaturation as indicated by an increased surface hydrophobicity. Thus, these irreversible alterations occurring following the exposure to high ionic strength combined with a lower pH could potentially offer an explanation for the reduction of the amount of water held within the myofibrils. The protein changes observed in myofibrils isolated from frozen-thawed meat could thus be mimicked in non-frozen meat by exposure to combinations of decreased pH and high ionic strength. Increased freezing-thawing cycles supported more severe denaturation of myofibrillar proteins indicated by reduced WHC and increased surface hydrophobicity in the isolated myofibrils (Fig. 1, Study III), which was, however, independent of the freezing rate. This observation is likely attributed to the repeated exposure of myofibrillar protein surface to pH-ionic strength combinations potentially occurring following additional freezing-thawing cycles. Therefore, it is speculated here that the freezing-induced protein changes in myofibrils, e.g. WHC, are likely attributed to the effect of a lower pH combined with concentrating ionic strength in the unfrozen liquid water rather than progressively concentrating ionic strength only.

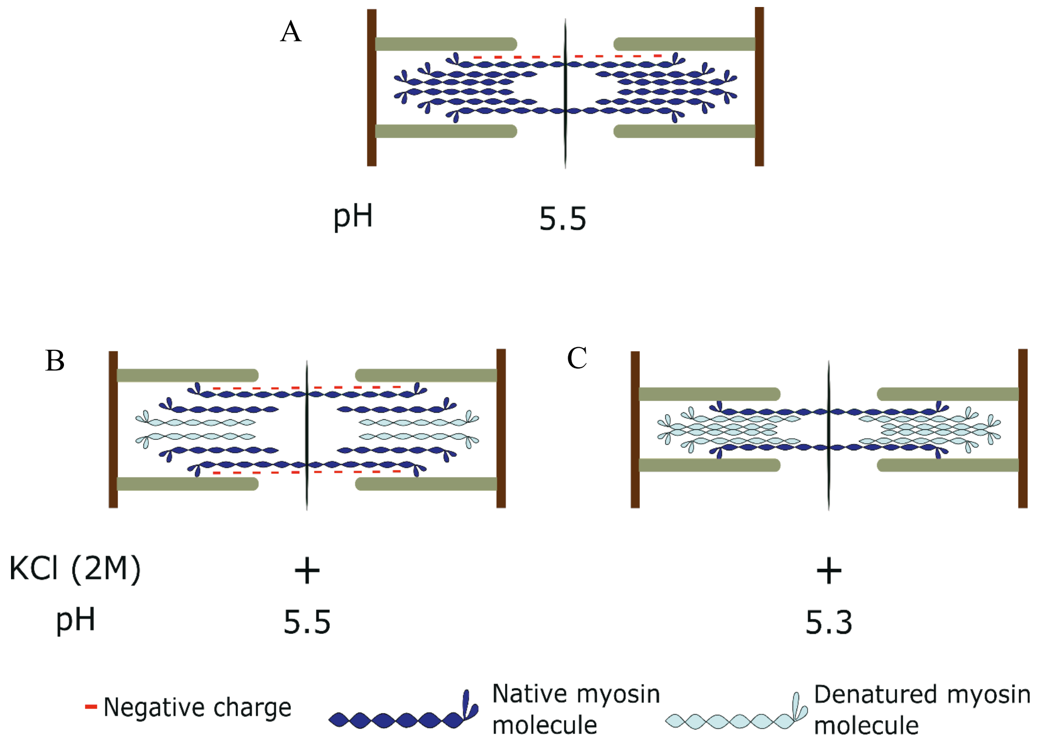

Fig. 7. Model illustrating possible events in freezing. The exposure to either high ionic strength (2 M KCl) only or high ionic strength (2 M KCl) combined with a lower pH (5.3) and the effect on myosin filaments within the sarcomere is shown. (A) Native sarcomere in fresh meat. Myosin molecules are 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 within the myosin molecules causing a swelling of myosin filaments, and consequently unit cell and the whole sarcomere, thereby increasing WHC. 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 (negative charges are not shown). KCl increases negative charges, however, the decrease of pH would cause a decrease in negative charges. Therefore, the combined effect results in reduced negative net charges of myosin filaments and transverse shrinkage of unit cell and thus the whole sarcomere, leading to reduced WHC. More myosin molecules are denatured by low pH and increased KCl concentration, 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. (Fig. 6, Study III).

6.3. Relationship between freezing rate and protein denaturation to explain the generation of thaw loss

The freezing rate has been reported to strongly affect the amount of thaw loss and slow freezing caused a larger water loss upon thawing compared to fast freezing (Fig. 2A, Study II; Fig. 1A, Study III; Fig. 1, Study IV). The size and location of ice crystals formed depending on the freezing rate have been regarded as the main explanation for the different amounts of thaw drip (Hamm, 1986; Li et al., 2018). The importance of myofibrillar protein denaturation in the generation of thaw loss is proposed in the current thesis. The freezing rate showed a distinct effect ($P < 0.05$) on the denaturation of myofibrillar proteins (Study II, III & IV), but this effect was not observed in the sarcoplasmic proteins (Study II). Therefore, it is speculated here that the difference in thaw loss in relation to the freezing rate is likely attributable to denaturation of myofibrillar rather than sarcoplasmic proteins.

Based on the observations that slow freezing resulted in more pronounced denaturation of myofibrillar proteins indicated by reduced WHC of myofibrils as well as increased surface hydrophobicity as compared to fast freezing, Study II introduces a model to explain the effect of myofibrillar protein denaturation in relation to the freezing rate on the generation of thaw loss (Fig. 8). The ice crystals usually are formed outside of muscle fibers at the beginning of freezing, which would cause an increase of solute concentration in the surrounding non-frozen water creating an osmotic pressure to allow the migration of water from inside of the muscle fibers to outside, thus contributing to dehydration and transverse shrinkage of the fibers (Koonz & Ramsbottom, 1939; Bevilacqua et al., 1979). Protons would then gradually accumulate with concentrating solutes and therefore a decline of pH is to be expected in the remaining non-frozen water. Temporary exposure to a lower pH combined with high ionic strength would hypothetically cause irreversible alterations in the myofibrils, like protein denaturation (Fig. 7). An increased dehydration of myofibrils and partial denaturation of myofibrillar proteins would then result in that less water will be reabsorbed upon thawing, thus increasing the amount of thaw loss.

The importance of decreased pH is to be expected to explain the mechanism of myofibrillar protein denaturation. It is to be speculated that the freezing rate could affect pH decline in the vicinity of myofibrillar protein surfaces during freezing of meat thus explaining the observed difference in myofibrillar protein denaturation and the resultant amount of thaw loss. Freezing at fast rate generates

numerous small ice crystals distributed uniformly within and between muscle fibers and additionally causes less transverse shrinkage of the fibers. In water, protons exist as the hydronium (H_3O^+) ions, and it has been predicted in both theoretical and experimental studies that bulk ice formed at low temperature could possibly trap excess protons (Park, Lin, & Paesani, 2014), although this has not been reported in frozen meat. It is thus postulated in the model (Fig. 8) that some protons might be trapped inside fast growing ice crystals formed in fast freezing, thereby causing reduced decline of pH occurring in the unfrozen water, and consequently less denaturation of myofibrillar proteins and reduced thaw loss observed in fast compared to slow frozen-thawed meat. However, slow freezing produces large ice crystals in the extracellular space following the migration of intracellular water to outside of the fibers, thus resulting in a more severe transverse shrinkage of the sarcomere as illustrated in Fig. 8. It is thus expected that higher concentrations of solutes and protons would occur in the remaining unfrozen water in slow freezing as compared to freezing at fast rate. Additionally, the exposure time of myofibrillar proteins to concentrating solutes and protons would be expected to be longer in slow compared to fast freezing. The corresponding lower pH combined with greater ionic strength in the remaining liquid water in slow freezing is thus hypothesized to induce more pronounced protein denaturation, and consequently a larger amount of thaw loss, as compared to fast freezing.

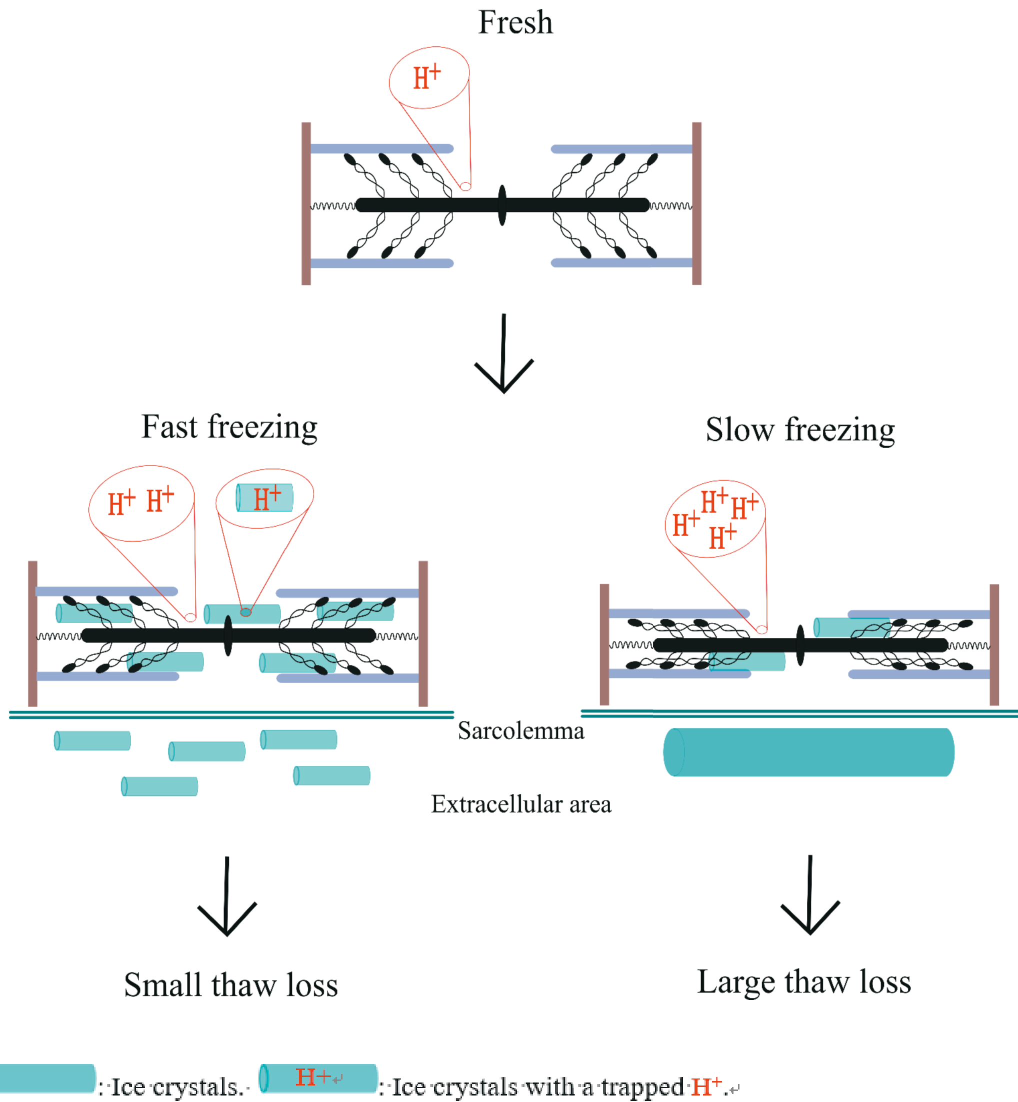

Fig. 8. Model explaining how the freezing rate affect pH of the unfrozen water phase within the sarcomere. In slow freezing migration of water to the extracellular area cause transversal shrinkage of the sarcomere and a higher concentration of solutes and protons in the remaining liquid water. Consequently pH declines within the myofibrils, resulting in pronounced pH and ionic strength induced protein denaturation, leading to reduced WHC and a high water loss upon thawing. In fast freezing small ice crystals could trap protons causing less reduction of pH in non-frozen water and hence lower amount of thaw loss when compared to slow freezing. H^+: the more H^+ in the diagram, the higher the concentration of protons within the sarcomere. (Fig. 4, Study II).

6.4. Effect of subzero temperature storage at -3 °C before thawing on the freezing-induced denaturation of myofibrillar proteins in fast or slow frozen pork

Freezing of muscle usually begins outside of muscle fibers as the temperature declines below -1 °C. The rate of protein denaturation in freezing is closely related to the subzero temperature (Buttkus, 1970), and it has been proposed in early studies that the maximum rate of protein insolublization reaction often occurs at a temperature range below the freezing point. As reviewed by Xiong (1997) and Sikorski et al. (1976), concentrating solutes in the unfrozen water with processing of freezing is responsible for the accelerated protein insolublization reaction, and in general, the lower the freezing temperature, the higher the solute concentration (Calvelo, 1981). Additionally, the influence of freezing temperature on the amount of unfrozen water within the meat tissue is to be expected and around 40% of the tissue water is unfrozen in beef at -3 °C, but only 10 % at -20 °C (Calvelo, 1981; Lee, Cornillon, & Kim, 2002). Considering the importance of water in chemical reactions, the further temperature lowering to reach the final frozen temperature could produce a counteractive effect and consequently reduce the rate of protein denaturation in freezing (Love & Elerian, 1964). The temperature at which there is a maximum rate of protein denaturation has been recognized by Calvelo (1981) and Finn (1932) as -3 °C in beef and by Love (1962) as -1.5 °C in cod. Muscle proteins have been shown to be denatured severely at the subzero temperature of around -3 °C. A decrease by around 10% of protein stability has been observed by Kaale & Eikevik (2016) in salmon after 3 days of storage at -1.7 °C, which was, however, similar to that observed in deep freezing for 30 days. In agreement, Sun et al. (2017) found a reduction of protein extractability by around 30% and loss of Ca^{2+}-ATPase activity by nearly 50% in swimming crab after being kept at -3 °C for 15 days. The hypothetical explanation for this phenomenon has been made mainly based on the effect of the progressively concentrating solutes occurring in the unfrozen water phase in parallel with removal of tissue water and temperature decline in freezing (Calvelo, 1981). However, a role of the pH decline in the remaining unfrozen water is hypothesized in this thesis (Fig. 7) to induce the denaturation of myofibrillar proteins occurring in freezing.

The freezing rate has been reported to affect myofibrillar protein characteristics (Wagner and Añón, 1985) and it is suggested in the model (Fig. 8) that when freezing at fast rate accumulating protons might be trapped inside fast growing ice crystals which might reduce the decline of pH in the unfrozen water within the meat tissue thus explaining the better myofibrillar protein characteristics when compared to freezing at a slow rate. However, the storage at -3 °C following deep frozen in Study IV

was observed to diminish the influence of the freezing rate on myofibrillar proteins. Accelerated protein denaturation was found with prolonged storage time at -3 °C as indicated by decreased WHC of myofibrils and increased surface hydrophobicity as well as earlier appearance of a myosin-4 fragment observed in SDS-PAGE. However, the initial differences of myofibrillar protein characteristics between fast and slow frozen groups became not evident after being stored at -3°C for 7 days. The pH of fast frozen-thawed meat was not significantly different with that of slow frozen-thawed meat (Fig. 2, Study IV), since the condition of reduced pH in the unfrozen water in freezing is believed as reversible when the unfrozen water gradually becomes diluted with the melting of ice crystals during final thawing at 2 °C. However, pH of frozen-thaw meat was observed to be lower ($P < 0.05$) both in fast and slow frozen groups followed by being kept at -3 °C for 7 days, presumably due to denaturation of buffer proteins and release of hydrogen ions that occurred at -3 °C (Leygonie at al., 2012). Together with the effects of the maximum denaturation at -3 °C this might diminish the positive benefit of the freezing rate on myofibrillar protein denaturation thus explaining the appearance of similar myofibrillar protein characteristics between fast and slow freezing groups after one week of storage at -3 °C.

Meat and meat products are often frozen to extend the shelf life in the industry, and fast freezing is preferably applied since it produces superior myofibrillar protein characteristics and less water loss upon thawing when compared to slow freezing. However, for further processing blocks of frozen meat usually requires time to be completely thawed, which is independent of freezing rate when considering the enormous quantity of frozen meat stored in the industry. The temperature of meat would then probably stay at around -3 °C for a relatively long period during the industrial thawing since the high latent energy of ice crystallization occurred within this temperature zone as suggested by Rahman et al. (2003). The conditions found under industrial thawing were studied in Study IV by investigating the influence of the subsequent storage at -3 °C on myofibrillar protein charactersitics in deep frozen pork treated with fast or slow freezing and a role of storage at -3 °C in diminishing the beneficial impact of fast freezing on myofibrillar protein attributes is suggested. Therefore, the meat industry should avoid excessive load during thawing in order to shorten as much as possible the temperature passage of -3°C.

7. Conclusions

Freezing-thawing treatment is shown to increase the free sarcoplasmic Ca^{2+} concentration of pork LTL muscle and to decrease extractable activities of calpain-1 and calpain-2 as well as increase activities of autolyzed products observed during the subsequent chilled storage. Around 50% of calpain-2 is activated after 9 days of frozen-then-chilled storage. Therefore, frozen-then-chilled storage can be a potential strategy to activate calpain-2 earlier postmortem, possibly leading to increased proteolysis. Proteasome activity is initially decreased by around 40% in the freezing-thawing process, and the activity is lower throughout the subsequent chilled storage in comparison with that of the non-frozen group, potentially indicating a negative effect of freezing-thawing on the proteasome-induced protein degradation.

A reduced WHC, increased surface hydrophobicity, as well as decreased transition temperature and denaturation enthalpy on DSC thermograms observed in the current thesis indicate myofibrillar protein denaturation occurring in the freezing-thawing process. Furthermore, sarcoplasmic protein denaturation is evidenced by a reduced denaturation enthalpy and fluorescence intensity observed in the purge drip collected from frozen-thawed pork muscle. Exposure of fresh meat to high salt (2 M KCl) increases WHC of myofibrils, whereas the involvement of decreased pH combined with high salt causes reduced WHC and increased surface hydrophobicity. This thesis thus suggests a role of decreased pH in combination with concentrating ionic strength in causing a denaturation of myofibrillar proteins that occurred by a comparable mechanism as conditions found in frozen-thawed meat.

The freezing treatment is shown to increase water loss upon thawing of pork LTL muscle, and freezing at slow rate causes an additional increase of thaw loss compared to freezing at fast rate. Additionally, slow freezing leads to a greater loss of WHC in parallel with an increase of surface hydrophobicity in the myofibrils, indicating that more pronounced denaturation of myofibrillar proteins is taking place in slow compared to fast freezing. However, these different parameters reach similar values when fast and slow deep frozen samples are subsequently stored at -3 °C for 7 days. The mechanism behind of the formation of thaw loss is proposed in a presented model principally being based on the involvement of myofibrillar protein denaturation in relation to the rate of freezing.

8. Future perspectives

The thesis showed that freezing-induced protein denaturation occurring in the myofibrils of pork LTL muscle as evidenced by reduced WHC, increased surface hydrophobicity and decreased denaturation enthalpy, and also in the sarcoplasmic fraction as assessed by denaturation enthalpy and fluorescence intensity. However, more indicators, e. g. changes of hydrogen and ionic bonds, are additionally required to better define the protein denaturation occurring in freezing-thawing process. The observations in this thesis suggest that an exposure of fresh meat and myofibrils to decreased pH combined with high KCl concentration rather than only high KCl concentration reduced WHC of myofibrils. This procedure may mimic the structural alterations within the myofibrils induced by the freezing-thawing process. More tests for this simulation experiment need to be provided. For instance, the influence on the lattice spacing between the filaments can be studied by X-ray diffraction measurement. Also the changes of water distribution and mobility within the muscle structure in relation to the combination of low pH and high ionic strength can be investigated by low-field NMR proton relaxometry.

The freezing rate produced a distinct effect on myofibrillar protein denaturation, and therefore a model of the influence of freezing-induced myofibrillar protein denaturation in the generation of thaw loss in relation to the freezing rate was proposed. It was speculated that fast growing small ice crystals might trap part of protons thus causing less decline of pH in the unfrozen water, and consequently less myofibrillar protein denaturation in fast compare to slow freezing. However, this speculation has not been proven in the current thesis. More research needs to be done to investigate the changes of pH in the unfrozen liquid phase in fast and slow freezing. For instance, ^{1}H and ^{31}P magic angle spinning NMR measurements can be applied to calculate the intracellular pH in the muscle tissue.

The subsequent storage at -3 °C for reflecting the condition of the industrial thawing led to accelerated denaturation of myofibrillar proteins in fast or slow frozen pork, and also introduced a negative impact on the WHC of meat. Therefore, how to shorten the time to thaw a large quantities of frozen block meat in order to avoid the temperature passage of -3 °C is of critical importance in the industry. The application of some modern techniques e. g. steam, microwaves, radiofrequency, ultrasound, high pressure can be a direction in the future.

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