Denaturation of myofibrillar and sarcoplasmic proteins in pale, soft and exudative-like meat:
Effects on water-holding

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ACADEMIC DISSERTATION

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Helsinki 2016
“... I took the one less traveled by, And that has made all the difference.”

Robert Frost
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Abstract
The aim of the thesis was to investigate the interaction between denatured sarcoplasmic and myofibrillar protein, considering the effects on the loss of water-holding in heat induced denaturation such as occurring in pale, soft and exudative (PSE) condition.

Porcine longissimus thoracis et lumborum muscles were incubated at temperature 0, 10, 20, 30 or 40 °C to 6 h post mortem. Incubation at 40 °C reduced the water-holding of meat ($P < 0.001$). SDS-PAGE and Western blot suggested that glycogen phosphorylase and creatine kinase precipitated onto the myofilaments, which was already accomplished at 6 h post mortem. Substantial meat tenderization was measured after incubation at 40 °C in parallel with less activity of extracted $\mu$- and m- calpains compared to lower temperatures, which indicated that an early activation of calpains by high temperature incubation could be the reason for the tenderization.

Surface hydrophobicity of myofibrils was higher ($P < 0.001$) after the pre-rigor incubation at 40 °C compared to lower temperatures. Less myosin subfragment-1 (S1) units were released by chymotryptic cleavage simultaneously with the loss of Ca$^{2+}$ ATPase activity after incubation at 40 °C. The results suggested that high temperature incubation induced microstructural alterations on the myosin head region, which may in turn be related to the loss of water-holding.

The role of the denatured sarcoplasmic proteins and myofibrils were compared. Myofibrils with sarcoplasm/protein-depleted sarcoplasm were combined and subjected to PSE-like condition. Water-holding was the poorest without the denatured sarcoplasmic proteins. Precipitated sarcoplasmic proteins shrank the myofilaments laterally by 6.3% after post-rigor incubation at 44 °C shown by X-ray diffraction. These results challenge the current understanding of the role of protein denaturation in water-holding, and therefore, a new hypothesis is proposed in this thesis: 1) within the intramyofibrillar space, the presence of precipitated sarcoplasmic proteins is associated with a compression in the lattice and thus expels water; 2) within the space outside of myofibrils (intermyofibrillar space) to the extracellular space, the coagulated sarcoplasmic proteins form a network that traps the water expelled from the intramyofibrillar space.
Preface

The current study was carried out in Meat Technology group, Department of Food and Environmental Sciences, University of Helsinki during 2013-2016. The work was financed by Kyllikki ja Uolevi Lehikoisen Säätiö; University of Helsinki, Meat Technology Research Funds; and University of Helsinki, Funds for completing thesis.

I want to give my special gratitude to my supervisor, Dr. Per Ertbjerg for always being supportive and inspiring throughout my entire doctoral study. I really appreciate all the discussion I had with you, which was enlightening, inspiring and led me to approach the goal in a more intelligent way. I would like to thank my co-supervisor, Professor Emeritus Eero Puolanne. Your scientific intelligence especially in water-holding motivated me to go deeper in this topic when I started. Apart from research, thank you both for always being there whenever I ran into troubles.

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Last but not the least, warmest thanks go to my families in my own language:

Helsinki, May 27, 2016.

Jiao Liu
List of original publications

Paper I

Paper II

Paper III

List of authorships
I and II
The study was planned by all the authors. The experimental analysis and manuscript preparation was done by Jiao Liu. Per Ertbjerg and Eero Puolanne gave comments and suggestions during manuscript preparation.

III
The study was planned by Jiao Liu, Per Ertbjerg and Eero Puolanne. Most of the experimental analysis was done by Jiao Liu. Low angle synchrotron X-ray diffraction measurements was done by Anders Arner. Manuscript preparation was done by Jiao Liu. Per Ertbjerg and Eero Puolanne and Anders Arner gave comments and suggestions during manuscript preparation.
Abbreviations

ANS 1, 8-anilino-naphtalene-sulfonate
ATP adenosine triphosphate
BisANS 4, 4'-dianilino-1, 1'-binaphthyl-5, 5'-disulfonic acid
BPB bromophenol blue
CPA cis-parinaric acid
DFD dark, firm and dry
DNTB 5, 5’-dithiobis-(2-nitrobenzoic acid)
DSC differential scanning calorimetry
EDTA ethylenediaminetetraacetic acid
ELC essential light chains
GM *gluteus medius*
HMM heavy meromyosin
LMM light meromyosin
LTL *longissimus thoracis et lumborum*
MES 2-(N-morpholino)ethanesulfonic acid
MOPS 3-(N-morpholino)propanesulfonic acid
MW molecular weight
NMR nuclear magnetic resonance
PCA protocatechuic acid
pH$_1$ pH measured at 45 min post mortem
pI isoelectric point
PM *pectoralis major*
PRODAN 6-propionyl-2-(dimethylamino)-naphthalene
PSE pale, soft and exudative
PSS porcine stress syndrome
RF *rectus femoris*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>RLC</td>
<td>regulatory light chains</td>
</tr>
<tr>
<td>S1</td>
<td>subfragment-1</td>
</tr>
<tr>
<td>S2</td>
<td>subfragment-2</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHG</td>
<td>second harmonic generation</td>
</tr>
<tr>
<td>SM</td>
<td><em>semimembranosus</em></td>
</tr>
<tr>
<td>ST</td>
<td><em>semitendinosus</em></td>
</tr>
<tr>
<td>Tris</td>
<td>tris(Hydroxymethyl)aminomethane</td>
</tr>
</tbody>
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1. Introduction

Lean fresh muscle contains around 75% of water. After slaughter some water will be lost from the meat by evaporation, exudation or cooking in the production line and during consumer’s home practice (Offer & Knight, 1988). The ability of meat to keep its natural or added moisture during processing is called water-holding capacity (Hamm, 1960) and it has been studied extensively as one of the most important meat quality attributes. Since meat is sold by weight, high drip loss will reduce the final product weight for selling and drip loss is thereby directly linked to the economy of producers. Drip contains around 120 mg/ml of protein (Offer & Cousin, 1992; Savage, Warriss & Jolley, 1990), which is often discarded by consumers. Substantial exudation on the surface of meat will be a minus to the appearance for the product, making it less appealing to the customers. In addition, the proteins lost along with drip are also a waste of animal proteins. Therefore, efforts have been made in the industry trying to maintain and to improve the water-holding capacity of meat after slaughter for the benefits of producers and consumers.

The drip loss of pork chops is typically about 2-5%, as reviewed by Fischer (2007). However, in pale, soft and exudative (PSE) meat in extreme conditions the drip loss can be as high as 12% by 48 h post mortem (Joo, Kauffman, Kim & Park, 1999). PSE meat is an example of quality deterioration, which is caused by either genetic defects in pigs or improper handling of the animals, slaughtering and chilling process. A carcass that is prone to develop PSE characteristics often has abnormally fast pH decline early post mortem, which enables the muscle to reach low pH (< 6.0) while the carcass is not chilled down yet (muscle temperature > 35 °C). Protein denaturation occurring in this situation is believed to be the crucial factor for the loss of water-holding (Bendall & Wismer-Pedersen, 1962; Fischer, Hamm & Honikel, 1979; van Laack & Kauffman, 1999). Indicators of protein denaturation have been found in PSE meat both in the sarcoplasmic and the myofibrillar protein fractions (Joo et al., 1999; Warner, Kauffman & Greaser, 1997).

The lean part of pork loin contains around 24% of protein, which is able to hold as much as 73% of water with fat content about 2% (Barbin, ElMasry, Sun & Allen; 2013; Correa et al., 2006; Okrouhla et al., 2008). This is achieved
by the highly organized hierarchy of the 3-D protein structures within the muscle fibre. Although PSE meat can be eliminated from the production line efficiently by gene selection and proper handling, protein characteristics within PSE meat should be studied carefully in order to understand the underlying fundamental mechanisms, i.e., how structural and soluble proteins interact with water molecules within the muscle structure. To date, different hypotheses intending to explain how protein denaturation relates to the loss of water-holding in meat have been proposed, yet the issue is still under debate (Huff-Lonergan, & Lonergan, 2005, 2007; Kim, Warner & Rosenvold, 2014; Kristensen & Purslow, 2001).

In the present thesis, pre-rigor and post-rigor incubation systems were set up to induce PSE-like conditions. By investigations of the characteristics of the soluble and structural proteins new information on the interaction of the protein matrix with water has been obtained. The ultimate goal is to increase the understanding of the causative relationships between protein denaturation and loss of water-holding in meat. This in turn will increase the theoretical knowledge of how water is retained within muscles.

At the beginning of the thesis (Chapter 2), the literature is reviewed: 1) introducing relevant physics and chemistry knowledge concerning the water distribution and mobility within muscle; 2) the PSE condition and a model system to mimic PSE condition in laboratory; 3) protein denaturation characteristics within PSE in relation with water-holding. The specific objectives of the present thesis are listed in Chapter 3. In Chapter 4, an outline of the materials and methods used will be presented while the more detailed descriptions are to be found from the original publications. In Chapter 5, a summary of the most important results will be depicted. Finally in Chapters 6, 7 and 8, a general discussion, conclusions, and future perspectives will be provided.
2. Literature review

2.1. Water-holding of muscle

2.1.1. Myowater distribution

Three quarters of the weight of lean muscle is water (Offer & Knight, 1988a), which is termed myowater and firmly retained within the muscle structure. It is important to understand the muscular microstructure in order to gain a comprehension on the compartments available for the accommodation of different water populations.

Skeletal muscle is composed of muscle fibres, which are long, tubular and multinucleated cells. A muscle fibre is composed of rod-like myofibrils, which in turn consist of myosin and actin filaments i.e., the thick and thin filaments, respectively. These structures are organized in a hierarchical way from macro to micro, which are: 1) muscle; 2) muscle fibre bundle (fascicle); 3) muscle fibre; 4) myofibril; 5) myofilament (Fig. 1).

Fig. 1. Illustration of skeletal muscle hierarchy

Thick and thin filaments are the fundamental units that build up the structure of myofibrils. If we zoom into one myofibril, repeated lateral dark and light stripes will be observed along the length. These striations constitute the repeated muscle contractile units named sarcomeres. The cross-sectional distances between two neighbouring thick filaments form a network called
lattice spacing (d) as shown in Fig. 5B. If a myofibril is illustrated as a simple cylinder, its total volume is determined by the length of the sarcomere and the lattice spacing.

Most of the living muscle volume is occupied by myofibrils. For instance, myofibrils can occupy up to 85% of the volume of a muscle fibre in guinea pig (Eisenberg & Kuda, 1975). As shown by Fig. 2, between adjacent myofibrils there are several subcellular constituents such as mitochondria and glycogen (Nielsen, Holmberg, Schröder, Saltin & Ørtenblad, 2011) and lipids. Nuclei in turn are situated between myofibrils and sarcolemma (Dubowitz, Oldfors & Sewry, 2013).

Since myowater takes up the biggest part of muscle mass, it is apparent that the majority of the water is held within the myofibrils i.e. within the space between the thick and thin filaments (intramyofibrillar space). The rest of water is held in the space outside of myofibrils. In detail, this space can be further divided into 1) between myofibrils (intermyofibrillar space) 2) between myofibrils and cell membrane (sarcolemma) 3) between muscle...
fibres and 4) between fibre bundles (Offer & Cousins, 1992). In total the amount of water within the myofibrillar protein matrix is approximately 85% and the remaining 15% is outside of myofibrils in muscle (Huff-Lonergan & Lonergan, 2005; Lawrie, 1998). Since water is a dipolar molecule, it also interacts with the charged surface of protein macromolecules and makes up a hydration shell. This water population is called the bound water as it is highly stabilized and resistant to external physical forces such as freezing and gravity (Fennema, 1996). However, these water molecules are not static. Instead, they are in fast exchange with the immobilized water surrounding the surface of protein macromolecules (Martini, Bonechi, Foletti & Rossi, 2013). Bound water constitutes only a small fraction of the total water amount, i.e., 0.5 g bound water/1 g of muscle proteins (Kuntz, 1974) making up about 8% of the fresh muscle weight. Therefore, bound water is not considered to be the water population where drip is originated (Huff-Lonergan & Lonergan, 2005). Besides the bound water, the rest, often referred to as the bulk water, is considered relatively “free” and susceptible to be expelled post mortem. The bulk water can be further divided into immobilized water and free water corresponding to the place where the water is held (Pearce, Rosenvold, Andersen, & Hopkins, 2011). Myowater is, as described above, held within different compartments, and the forces that exist within each distinct environment influence the easiness of water removal as shown in Table 1.
Table 1. Summary of water compartmentalization within muscle.

<table>
<thead>
<tr>
<th>%</th>
<th>Compartment</th>
<th>Scale</th>
<th>Main force that may be involved</th>
<th>Strength</th>
<th>Can be mobilized? If Yes, by:</th>
<th>(^1\text{H NMR T2 relaxometry}^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Bound water</td>
<td>Å</td>
<td>Hydrogen bond</td>
<td>Strongest</td>
<td>No T(_{2b})</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>Immobilized (entrapped) water</td>
<td>(d_{1b}: 39-42 \text{ nm})</td>
<td>Steric force, Electrostatic force, Osmotic force, Constrains from transversal structural elements</td>
<td>Intermediate</td>
<td>Rigor formation, Rigor formation</td>
<td>(T_{2\text{i}})</td>
</tr>
<tr>
<td>15</td>
<td>Free water</td>
<td>0.2-0.5 (\mu\text{ m})</td>
<td>Capillary force</td>
<td>Weakest</td>
<td>Gravity</td>
<td>(T_{2\text{j}})</td>
</tr>
</tbody>
</table>


Superscript \(^4\): Low field (LF)-NMR relaxometry of \(^1\text{H}\). The distributed exponential fitting analysis of NMR T\(_2\) relaxation data revealed the presence of three distinct water populations (T\(_{2b}\), T\(_{2i}\), T\(_{2j}\)) (Bertram, Andersen & Karlsson, 2001).
2.1.2. Myowater mobility

What sorts of forces then are able to hold the bulk water within the myofibrillar protein system? The hypothesis from Hamm (1972) emphasized that the electrostatic repulsive force between the filaments are the driving force to swell the system. Offer and Knight (1988a) stressed the structural aspects of the lattice in the myofibrils, proposing that the selective binding of ions to the filaments will create an osmotic pressure that will swell the myofibrils. Based on these classical hypotheses, the effects of adding salt, changing pH, and protein denaturation, can all be well explained. Although it is difficult to find a fundamental explanation for bulk water holding in muscle, Puolanne and Halonen (2010) stressed that the interaction between protein surface and the surrounding solution is very important. Moreover, the effects of ions and proteins on water structures were explained in detail. The transversal elements bridging the filaments, such as the heavy meromyosin, are very important for water-holding since they represent 2/3 of the water accessible surface of structural proteins within a myofibril (Puolanne & Halonen, 2010). Offer and Trinick (1983), Hermansson (1986) and further supported by Tornberg (2005) have suggested that bulk water is held by capillary forces. However, it is hard to imagine the existence of a capillary structure within the myofibrillar lattice, where the prevailing actomyosin bonds create and keep up spatial interference between the filaments.

Low field (LF)-NMR relaxometry is a non-invasive technique that has been used intensively as a tool to study the myowater distribution and mobility within the muscle and meat matrix. Nuclei such as $^1$H in water molecules are aligned in a magnetic field and subjected to a radio frequency pulse. After absorbing the oscillation energy and the subsequent cessation of the pulse, the $^1$H will return to its original state by a characteristic time that is called the relaxation time, which depends on the stability of the $^1$H. By measuring this relaxation time, it is, therefore, possible to reflect the physical/chemical state
of $^1$H and thereby provide information on the myowater mobility compartmentalization. The relaxation occurs in the longitudinal vector ($T_1$) and the transversal vector ($T_2$) (Betram, Schäfer Rosenvold & Andersen, 2004). The distributed exponential fitting analysis of NMR $T_2$ relaxation data has revealed the presence of three distinct water populations ($T_{2b}$, $T_{21}$, $T_{22}$) (Bertram, Andersen & Karlsson, 2001) as shown in Table 1. $T_{2b}$ is the fastest of the three relaxation times and thus highly likely to represent the water population ($P_{2b}$) bound to macromolecules such as proteins (1–4%). The major water population $P_{21}$ represented by $T_{21}$, the intermediate relaxation time, is speculated to reflect the water entrapped between the myofilaments (85–96%). The slowest relaxation time $T_{22}$ is believed to represents the water population ($P_{22}$) of the extrafibrillar space (2–10%) (Betram & Andersen, 2004; Bertram et al., 2001).

The $T_2$ relaxation time is affected by the structure of the myofilament lattice as well as the pH decline post mortem (Tornberg, Wahlgren, Brøndum & Engelsen, 2000). Bertram, Purslow and Andersen (2002b) observed an increase in $T_{21}$ relaxation time in stretched sarcomeres, which could be explained by the less protein density in I-band compared to that in the A-band. In addition, the $T_{21}$ relaxation time reflects the muscle’s type and its growth stage (Bertram et al., 2002c). Furthermore, Tornberg et al. (2000) found that $T_{21}$ and $T_{22}$ as well as $P_{21}$ and $P_{22}$ were influenced by the post-mortem pH decline rate. An increase in $P_{21}$ suggests an increase in the immobilized water within the intramyofibrillar space. The $P_{22}$ population has been shown to be highly correlated with water-holding capacity, suggesting that water loss originates from the “free water” that is loosely held in the extrafilamental space (Bertram et al., 2002a; Bertram, Dønstrup, Karlsson & Andersen, 2002a). Therefore, it appears that NMR $T_2$ relaxation is an effective technique to obtain information on the relationships between muscle structure, water distribution and mobility, and water-holding capacity.
2.1.3. Factors affecting water-holding during the conversion of muscle to meat

Good water-holding capacity has positive impacts on the sold weight of fresh meat, as well as the colour, juiciness and tenderness of cooked meat, etc. as stated in the Introduction. However, after the animal has been slaughtered, the myowater gradually migrates from the intracellular to the extracellular space during the conversion of muscle to meat.

Most of the water population is entrapped between the myofilaments. Therefore, factors that induce shrinkage of the sarcomere volume will indirectly influence the amount of water that can be held. In the living state muscle contraction is under isovolumetric condition over a wide range of sarcomere lengths (Millman, Racey & Matsubara, 1981) so the intracellular water will not be expelled to extracellular space upon the shortening of the sarcomere. However, during the conversion of muscle to meat, permanent rigor bonds start to form, which will gradually lock the adjacent thick and thin filaments. Any microstructural changes occurring within the sarcomere architecture that will shrink the muscle fibre in volume will, thereby, have an effect on the water distribution and mobility. Consequently, drip will originate from the extracellular fluid that in turn has migrated from the intracellular space (Pearce et al., 2011).

2.1.3.1. Steric effect

After death of the animal, the muscle starts to convert into meat. Several fundamental biochemical processes, such as anaerobic glycolysis, will proceed within the tissue resulting in a subsequent pH decline, and later, the leakage of Ca\(^{2+}\) ions from the sarcoplasmic reticulum to the sarcoplasm. Sarcomeres contract in the presence of ATP and Ca\(^{2+}\), which induces a longitudinal shortening of myofibrils. The rigor shortening of myofibrils has been suggested to increase the migration of intramyofibrillar water to the extrafilamental space (Tornberg et al., 2000). The shortening of sarcomeres
proceeds during rigor development until no ATP is available in the sarcoplasm. Sarcomere lengths also depend on the pre-rigor temperature. Maximum shortening has been observed at -2 and +38°C, and the minimum at +10 °C along with a corresponding minimum drip loss (Honikel, Kim, Hamm & Roncales, 1986; Locker & Hagyard, 1963). Manipulation of sarcomere length e.g., by stretching the sarcomeres after rigor completion, has been shown to reduce the drip loss (Hughes, Oiseth, Purslow and Warner, 2014).

In addition, sarcomeres shrink laterally due to the formation of actomyosin and the drop of pH. Using electron microscopy, Offer and Cousins (1992) observed a gradual lateral shrinkage of myofibres with a subsequent enlargement of space between fibres as well as fibre bundles from 2-48 h post mortem. Early post mortem a decrease in lattice spacing has also been shown by X-ray diffraction measurements (Diesbourg, Swatland, & Millman, 1988; Guignot, Vignon & Monin, 1993; Offer et al., 1989) due to the formation of actomyosin bonds between thick and thin filaments post mortem (Goldman, Matsubara & Simmons, 1979; Offer & Knight, 1988), and pH decline (Diesbourg et al., 1988; Irving, Swatland, & Millman, 1990). If the pH declines very fast post mortem, the combination of low pH and high temperature will likely denature the myofibrillar proteins, which shrinks the lattice transversally and thereby reduces the space for water to be held (Offer & Knight, 1988b). This part will be discussed in the section 2.3.

2.1.3.2. Net charges effect

Myofilaments are composed of amino acids. Water molecules are pulled by the polar groups and pushed by the none-polar groups of side chains of the amino acids (Hamm, 1972; Ling, 1965). The combined effect is believed to be the early thought of kosmotropic effect as recently introduced by Puolanne and Halonen (2010) in more detail. The charged side chains exposed on the surface gives the protein net charges. The isoelectric point (pI) of the
myofibrillar proteins, at which the water-holding capacity is at its minimum, is around 5.0 (Hamm, 1972). Post mortem, when pH starts to decrease from 7.0 and approach the pI of myofibrillar proteins, the net charges on the filaments decrease and consequently the sarcomeres shrink laterally (Hamm, 1972; Offer & Knight, 1988b). Offer and Knight (1988) claimed that the distances between the adjacent filaments are too long to establish a repulsive force strong enough to create a swelling pressure. Nevertheless the electrostatic forces between the polar groups and water molecules on the surface of proteins, and between water layers, create very important surface interaction, which in turn has impact on the water-structure and therefore explains the water-holding within muscle (Puolanne & Halonen, 2010).

Lattice spacing was shown to increase corresponding to pH changes from 5.2 to 6.4 by X-ray diffraction (Irving et al., 1990). Low pH combined with relatively high temperature will also precipitate some of the sarcoplasmic proteins, negatively affecting the water-holding of myofilaments by shielding of the net charges (Bendall & Wismer-Pedersen, 1962). Unlike the impact of pH, that of denaturation is irreversible. This part will be reviewed in details in section 2.3.

2.1.4. Formation of drip channels

The extracellular space of a muscle fibre is in its minimum size in the living state. However, the extracellular space will be gradually enlarged, leaving gaps between neighbouring fibres and fibre bundles which will eventually open channels for intracellular sarcoplasm to drip out (Hughes et al., 2014; Offer & Cousins 1992). The reduction in the volume of myofibrils is the initial driving force for water to start migration (Offer et al., 1989). However, water that is expelled from myofilaments will not move to the extracellular space immediately. Instead, water will enter the space between myofibrils and sarcolemma first and then drain out slowly (Schäfer, Knight, Wess & Purslow, 2000). Therefore, the time frame of myowater migration from
intramyofibrillar $\rightarrow$ extramyofibrillar $\rightarrow$ extracellular spaces is very important since it is an ongoing process post mortem that determines the amount of water susceptible to be lost as drip thereafter.

Drip originates from sarcoplasm as it has the same protein composition and in same amount as in sarcoplasm (Savage et al., 1990). It has been proposed that drip originates from myofibrils due to the lateral shrinkage (Diesbourg et al., 1988; Offer & Knight, 1988b). Studies have provided evidence that the shrinkage of the lattice spacing will in turn shrink the fibre laterally and later on increases the extracellular space (Guignot et al., 1993; Honikel et al., 1986; Offer & Cousins, 1992). By light-microscopy it was suggested that drip formation is a two-stepped process (Offer & Cousins, 1992). At first, moisture migrates from the intramyofibrillar space to the extracellular space, from which it gets squeezed out onto the surface of meat. Offer & Cousins (1992) showed in the microscopy images that at 2 h post mortem bovine muscle fibres still filled up the endomysial and fibre bundles the perimysial network. At 4-6 h, due to the lateral shrinkage of myofibrils and their connection with sarcolemma, gaps started to appear in between fibre bundles in the perimysial network but not in the endomysium network. Finally, at 24-48h, gaps between fibres were also clearly detectable. However, Schäfer, Rosenvold, Purslow, Andersen, and Henckel (2002) showed that space between bundles decreased gradually during 9 to 24 h post mortem, and they speculated it was sacrificed by the continuing enlargement of the gaps between fibres. As a result of the enlarged gaps between fibres, drip channels are created, where water plus sarcoplasmic proteins will be lost by gravimetric force etc. (Offer et al., 1989). Pork loins with substantially higher drip loss had even earlier the onset of drip channel formation compared to the normal ones (Hughes et al., 2014). Water compartmentalization shown by NMR T₂ relaxometry is in agreement with this process. Increased population in T₂₂ and simultaneous decreased population in T₂₁ are shown during the rigor development (Bertram & Andersen, 2004; Tornberg et al., 2000).
migration of water (sarcoplasm) to the extracellular space will not be feasible without passing through the sarcolemma. During the development of rigor mortis, sarcolemma loses its integrity, and this is accompanied by an increase in water leaking from the intramyofibrillar to the extracellular space (Bertram et al., 2002c). Therefore, also the permeability and/or breakage of the sarcolemma has an influence on the amount of drip that will be created.

2.2. PSE and PSE-like condition

Pale, soft and exudative (PSE) meat is a well-known meat quality defect. PSE meat is characterized by pale colour with a substantial amount of drip loss. According to Joo, Kauffman, Kim and Park (1999), PSE cases from mild to severe, have drip losses ranging from 8.6 to 11.6% by 48 h post mortem, while those of normal meat stay below 6%.

Among the studies on water-holding related topics, PSE meat is often used as a negative example to investigate the difference in e.g. pre-rigor physical condition, biochemical and structural characteristics that may explain the loss of water-holding capacity in meat. These studies are intended to provide solutions to prevent the development of PSE meat in meat production.

2.2.1. Mechanisms behind PSE development

Pigs carrying the halothane gene are very susceptible to developing porcine stress syndrome (PSS) when exposed to stress during transportation or pre-slaughter handling, and/or to the anaesthetic gas halothane (Rønvold & Andersen, 2003b). The halothane gene is a mutated gene (Ryr1) encoding the ryanodine receptor isoform. With this gene mutation, the ryanodine receptor/Ca\(^{2+}\) release channels within the sarcoplasmic reticulum of a muscle fibre fail to control the release of Ca\(^{2+}\) when experiencing stress (Fujii et al., 1991; O’Brien, 1986). The increasing amount of sarcoplasmic Ca\(^{2+}\) will result in an extensive consumption of ATP in sarcomeres as well as an accelerated pH decline with a subsequent change in metabolism (Barbut et al., 2008), which is closely related to the occurrence of PSE characteristics. If the pigs...
having the homozygous halothane gene do not die of physical stress, their odds of developing PSE meat is increased by over 80%, even with the best of handling (Lee & Choi, 1999). The halothane test is only able to identify homozygous halothane genotype (nn) from halothane gene carrier (Nn) and non-carrier (NN), but not between Nn and NN. However, although not as severe as nn-pigs, Nn-pigs are also susceptible to have an abnormally rapid glycolysis after exposure to stress (Lundström, Essen-Gustavsson, Rundgren, Edfors-Lilja & Malmfors, 1989). Therefore, a DNA test has been used in order to eliminate the halothane gene from the breeding lines in many countries, such as the US (Huff-Lonergan & Lonergan, 2005), Denmark, Netherland, Sweden and Switzerland (Rosenvold & Andersen, 2003b). The RN- gene (Rendement Napole gene) is another one resulting in lighter meat with a higher drip loss (Deng et al., 2002; Le Roy et al., 2000). RN- gene carrying pigs have exceptionally high production of glycogen in muscle, and therefore more protons will be produced in glycolysis (Estrade, Vignon & Monin, 1993; Monin & Sellier, 1985). The ultimate pH is lower in these pigs (Faustman & Cassens, 1990), and that too results in lowered water-holding (Le Roy et al., 2000).

Even without the halothane gene, pigs are very sensitive to physical stress. Environmental and management factors, such as ambient temperatures, feeding regimen, animal mixing, and transportation, may all withhold negative and potentially PSE-inducing effects, as reviewed by Lee and Choi (1999). Short-term stress prior to stunning will accelerate the metabolism in muscles, which result in a rapid pH decline (van der Wal, Engel & Reimert, 1999; Rosenvold & Andersen, 2003a). This condition is not as severe as that caused by halothane gene defect, yet may still result in PSE meat development (D’Sousza, Dunshea, Warner & Leury, 1998).

However, even with careful animal handling applied to avoid pre-slaughter stress in pigs, PSE meat may still manifest itself due to inefficient carcass chilling and the consequently high temperatures within deep muscle
locations (Bendall & Swatland, 1988; Maribo, Olsen, Barton-Gade, Møller, & Karlsson, 1998; Rosenvold & Andersen, 2003b). Glycolysis is responsible for the drop of pH, which is an anaerobic enzymatic process. Thus the speed remains relatively fast when the carcass is not yet chilled down efficiently early post mortem (Offer, 1991).

As discussed above, genetic defects and mishandling will result in a similar consequence, the abnormally fast pH decline post mortem. It has been summarized that muscle with a pH < 6.0 at 45 min (pH1) post mortem is very likely to develop PSE characteristics (Adzitey & Nurul, 2011). In non-PSE carcasses, the pH decline rate is around 0.01 units/min, whereas that of PSE carcasses is 0.02 units/min. In extreme PSE, the rate can be as high as 0.1 units/min (Offer, 1991). A living animal has a body temperature of about 39 °C. Shortly after slaughter the temperature will first rise to almost 42 °C even in normal carcasses (Schäfer et al., 2002) but then gradually decline depending on chilling conditions. With more rapid glycolysis the muscle temperature would shortly rise even more. Thus, in extreme PSE carcasses, the temperature will, however, be able to reach as high as 44-45 °C within a few minutes after death (Ludvigsen, 1954) cited by Offer (1991). When pH declines at a very fast speed, the muscle will enter a period of time when the pH is < 5.7, while the temperature is still above 35 °C. The combination of high temperature and low pH will induce protein denaturation within the muscle, which has been widely accepted to be the main factor behind the loss of water-holding, increase in colour paleness, as well as to have various other deleterious quality effects (Bendall & Wismer-Pedersen, 1962; Fischer et al., 1979; Offer & Trinick, 1983; van Laack & Kauffman, 1999). For a recent review covering different animal species, see Kim et al. (2014).

2.2.2. Model system to mimic PSE condition

Selecting PSE muscles at the slaughter line is often based on colour, pH1 and drip loss (Adzitey & Nurul, 2011). Although there are many papers providing
exact criteria for selecting PSE, it is sometimes difficult to decide which ones to follow since the criteria differ depending on plant conditions and researchers (Lesiow & Xiong, 2013). A standardized method to develop meats with different quality properties would therefore be beneficial as it would help to obtain consistent PSE-like meat for experimental purposes.

2.2.2.1. Pre-rigor temperature incubation

In order to manipulate the pH decline rate before rigor is completed, the easiest way is perhaps an early post mortem incubation of muscle in altering environmental temperatures. Glycolysis involves a series of enzymatic steps. One of the key attributes to determine the activity of the enzyme is temperature. The initial glycogen content provides the fuel for glycolysis, and when glycogen is gradually used up, the speed of glycolysis will slow down as well. Also the initial level of glycogen may, therefore, influence the pH decline rate. However, according to Klont, Talmant & Monin (1994) and Maribo et al. (1998), the early post mortem metabolic rate is independent on the glycolytic potential and mainly dependent on the incubation temperature. By raising the temperature from 38 to 42 °C, the rate of pH decline was almost doubled, although the temperature effect on the pH decline rate was not as profound as observed in the PSS case (Klont et al., 1994). Therefore, if the initial glycogen level is within a normal range, it is possible to mimic the rapid pH decline as in PSE meat. Successful examples to induce PSE-like characteristics including protein denaturation, paleness in colour and high drip loss have been obtained in pork (Brewer, Zhu, Bidner, Meisinger & McKeith 2001; Fernandez, Forslid & Tornberg, 1994; Lesiow & Xiong, 2013; McCaw, Ellis, Brewer & McKeith, 1997), turkey (Mckee & Sams, 1998; Zhu et al., 2013) and chicken (Zhu et al., 2011) muscles where pre-rigor incubation of temperatures ranging from 35 to 40 °C for 4–7 h was introduced.
2.2.2.2. Post-rigor temperature incubation

Since pre-rigor incubation requires the harvesting of a muscle as soon as possible from the hot carcass at the slaughterhouse, a more practical and yet applicable approach is the use of post-rigor meat. Freise, Brewer and Novakofski, (2005) were able to replicate similar paleness and drip loss to pork chops as in PSE meat, by incubating post-rigor pork chops with pH < 5.8 at 42 °C for 1 h. Temperatures below 34 °C had no effect on the colour of post-rigor pork chops. To obtain more specific information, post-rigor incubation may also be applied on purified myofibrils. By incubating myofibrils in a buffer at pH around 5.4 and temperature around 40 °C it has been possible to induce PSE-comparable denaturation to myofibrils extracted from muscle of rabbit (Penny, 1967b) and pig (Monin & Laborde, 1985; van Laack & Lane, 2000), but not from poultry breast muscle (van Laack & Lane, 2000).

2.3. Protein denaturation in relation to water-holding

After water, protein is the second largest component in fresh meat (Lawrie, 1998). By solubility, proteins can be divided into the soluble fraction of the sarcoplasmic proteins and the insoluble fraction of the structural proteins i.e. myofibrillar proteins. Around 50-55% of the total protein entity is made up of the myofibrillar, and 30-34% of the sarcoplasmic proteins (Helander, 1957; Morissey, Mulvihill & O’Neill, 1987). Evidence showing that denaturation occurs in the sarcoplasmic and myofibrillar proteins in PSE meats from different species is summarized in Table 2. Denaturation characteristics have been investigated by different methodologies, and additionally several hypotheses have been proposed by different studies in order to explain the mechanisms behind the loss of water-holding capacity in PSE meat (Bendall & Wismer-Pedersen, 1962; Joo et a., 1999; Warner et al., 1997; Offer, 1991; Offer & Knight, 1988a, 1988b; Zhu et al., 2011; van Laack, Liu, Smith & Loveday, 2000).
**Table 2.** Summary of evidence indicating the sarcoplasmic and myofibrillar protein denaturation occurring in meat with PSE characteristics from literature.

*GM gluteus medius; LTL longissimus thoracis et lumborum; PF pubo-ischio femorale; PM pectoralis major; PP pectoralis profundus; RF rectus femoris; SM semimembranosus; ST semitendinosus.*

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<tr>
<th>Fraction</th>
<th>Denaturation indicator</th>
<th>Muscle</th>
<th>Reference</th>
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| Myofibrillar protein | Reduced solubility /extractability | Pork LTL | Wismer-Pedersen, 1959  
Bendall & Wismer-Pedersen, 1962  
Sayre & Briskey, 1963  
Scopes, 1964  
Penny, 1969  
Lopez-Bote, Warriss & Brown, 1989  
Boles, Parrish, Huiatt & Robson, 1992  
Warner, Kauffman & Greaser, 1997  
Joo, Kauffman, Kim & Park, 1999  
Ryu, Choi & Kim, 2005 |
| | | Pork SM PM | van Laack & Lane, 1999 |
| | | Boiler PP PF | van Laack & Lane, 1999 |
| | | Bovine LTL | van Laack & Lane, 1999 |
| | | Pork LTL | van Laack & Lane, 1999 |
| | | Bovine LTL | van Laack & Lane, 1999 |
| | | Bovine LTL | Penny, 1969  
Sung, Ito & Izumi, 1981  
Warner et al., 1997  
Liu, Puolanne & Erbjaerg, 2014a |
| | | Lamb GM SM ST | Warner, Kerr, Kim & Geesink, 2014 |
| | | Rabbit myofibrils/myosin | Penny, 1976a  
Penny, 1967b |
| Changes in swelling curve upon pH | Pork LTL | Bendall & Wismer-Pedersen, 1962  
Hamm, 1977 |
| | | Pork LTL | Stabursvik, Fretheim, & Frøyestead 1984  
Deng et al. 2002 |
| | | Pork LTL | Liu et al., 2014a |

*(Continued next page)*
Table 2. (Continued)

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<th>Fraction</th>
<th>Denaturation indicator</th>
<th>Muscle</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Sarcoplasmic protein |                       | Pork LTL | Sayre & Briskey, 1963  
|              |                        |         | Fischer, Hamm & Honikel, 1979                                             |
|              |                        |         | Lopez-Bote et al., 1989                                                   |
|              |                        |         | Boles et al., 1992                                                        |
|              |                        |         | Ryu et al., 2005                                                          |
|              |                        |         | Liu, Ruusunen, Puolanne & Ertbjerg, 2014b                                |
| Reduced solubility |                       | Broiler PM | van Laack, Liu, Smith & Loveday, 2000                                     |
|              |                        |         | Zhu, Ruusunen, Gusella, Zhou & Puolanne, 2011                            |
|              |                        |         | Bowker & Zhuang, 2015                                                     |
|              |                        | Lamb GM  | Warner et al., 2014                                                       |
|              |                        | SM      | Warner et al., 2014                                                       |
|              |                        | ST      | Warner et al., 2014                                                       |
|              |                        |         | Warner et al., 2014                                                       |
|              |                        |         | Warner et al., 2014                                                       |
|              |                        |         | Warner et al., 2014                                                       |
|              |                        | Broiler PM | Zhu et al., 2011                                                          |
|              |                        |         | Bowker & Zhuang, 2015                                                     |
|              |                        | Bovine LTL | den Hertog-Meischke et al., 1996                                         |
|             | Enzyme precipitation by electrophoresis | Pork LTL | Borchert, Powre & Briskey 1969                                             |
|              |                        |         | Warner et al., 1997                                                       |
|              |                        |         | Joo et al., 1999                                                          |
|              |                        |         | Ryu et al., 2005                                                          |
|              |                        |         | Liu et al., 2014b                                                         |
|              |                        | Broiler PM | Zhu et al., 2011                                                          |
|              |                        |         | Bowker & Zhuang, 2015                                                     |
|              |                        | Bovine LTL | den Hertog-Meischke et al., 1996                                         |
|             | by Western blot        | Pork LTL | Liu et al., 2014b                                                         |
|              |                        |         | Liu et al., 2014b                                                         |
|              |                        | Broiler PM | Zhu et al., 2011                                                          |
|              |                        |         | Bowker & Zhuang, 2015                                                     |
|              |                        | Turkey PM | Pietrzak, Greaser & Sosnicki, 1997                                       |
|             | Immunofluorescent blotting | Turkey PM | Pietrzak et al., 1997                                                    |
|             | Reduced enzyme activity | Pork LTL | Fischer et al., 1979                                                     |
|             | Changes in hydrophobicity by bisANS | Pork LTL | Gratacós-Cubarsi & Lametsch, 2008                                        |
|             | DSC                    | Pork LTL | Deng et al. 2002                                                         |
2.3.1. Myofibrillar protein denaturation

2.3.1.1 High temperature denaturation

Although with the PSE condition, temperatures above 45 °C are not involved, an understanding of the high temperature denaturation of myofibrillar proteins, e.g. in cooking conditions, may also shed light on the mechanisms behind the myofibrillar protein denaturation at lower temperatures.

From a polypeptide to an entire protein macromolecule, functionality will not be achieved without the primary, secondary, tertiary and sometimes quaternary structures. In muscle, proteins build up a fibrous structure: thick and thin filaments along with the whole myofibrillar protein entity assemble themselves into a network of α-helixes and β-sheets. The stabilization of the protein structure is dependent on non-covalent forces i.e. hydrogen bonds, electrostatic forces, hydrophobic interactions, and van der Waals forces. The loss of quaternary, tertiary and secondary structures caused by application of external energy, e.g. heating, is defined as heat denaturation. Heat denaturation of muscle proteins has been extensively studied since it is relevant to the functionality of cooked meat products. The specific temperatures where different proteins start to denature have most often been investigated by differential scanning calorimetry (DSC). One of its advantages lies in the applicability for a complex biological mixture; protein fractionation and purification are thus not required.

DSC studies in muscles from different species have revealed thermograms with three distinctive peaks as illustrated in Fig. 3 (Deng et al., 2002). Peak I represents the denaturation of the myosin head, Peak II represents that of the myosin rod as well as the sarcoplasmic proteins, and finally, Peak III reveals actin denaturation (Deng et al., 2002; Wright, Leach & Wilding, 1977; Xiong, Brekke & Leung, 1987).
Fig. 3. A typical DSC thermogram of muscle that is composed of three major peaks. I: Myosin heads; II: Sarcoplasmic proteins and myosin tails; III: actin (Deng et al., 2002).

In the circumstance of cooking whole meat, the fibrous filaments will tend to shrink upon heat denaturation while globular proteins, such as sarcoplasmic proteins, will tend to unfold, as reviewed by Tornberg (2005). The shrinkage of fibres takes place both longitudinally and laterally. The lateral shrinkage of muscle fibres occurs at a lower temperature (~ 40 °C) than that of connective tissue (~ 60 °C), and therefore cooking continues widening the gaps that already existed between fibres after rigor (Tornberg, Andersson & Josell, 1997). The mechanical force created by myofibrillar shrinkage first pushes moisture into the extracellular space. Then, as the temperature rises up to and above 60 °C, the subsequent shrinkage of collagen will cause the extracellular water to be expelled as cooking loss. This process of cooking loss formation was first described by Offer (1984), and then demonstrated further by Tornberg & Larsson (1986) with the use of NMR T2 relaxometry.

More recently, the heat induced structural changes in the fibrous structures of muscle was real-time-studied using the method of second harmonic generation (SHG) microscopy (Brüggemann, Brewer, Risbo & Bagatolli, 2010).
In agreement with DSC, SHG microscopy shows that the heat denaturation occurs in myofibrillar proteins mostly at lower temperatures than in collagen.

Prolonged heat treatment at low temperature is an alternative method of cooking to obtain better juiciness and tenderness in pork (Christensen, Ertbjerg, Aaslyng & Christensen, 2011; Christensen, Bertram, Aaslyng & Christensen, 2011). The studies on this topic included conditions even closer to PSE condition compared to conventional cooking, since meat is always cooked at the ultimate pH with temperatures often in the range from 48 to 57 °C for around 6 h or longer. Therefore, cooking at lower temperature e.g. 48 °C for several hours may be speculated to denature the myofibrillar proteins in a way sharing similarities with the PSE scenario.

2.3.1.2. Evidence in PSE

*Extractability and solubility*

The myofibril is composed of an insoluble protein structure. The protein solubility and extractability have been used as indicators of the myofibrillar protein denaturation occurring in PSE meat. The extractability of myofibrillar proteins can be determined by measuring the amount of myofibrillar proteins that is solubilized by a buffer of high ionic strength (above 0.3 M), extreme pH or with special chemicals (Scopes, 1964; van Laack & Lane, 2000; Zayas 1997). Alternatively the solubility of myofibrillar proteins can be measured by first solubilizing total proteins from the sample and determination the protein content, and then deducting the content of sarcoplasmic proteins (Warner et al., 1997; Joo et al., 1999; Bowker & Zhuang, 2015).

Scopes (1964) reported that after being kept at 37 °C for 4 h early post mortem, the extractability of myofibrillar protein decreased by around 50%. Later Warner et al. (1997), Joo et al. (1999) and Ryu et al. (2005) found a significantly lower myofibrillar protein solubility in PSE pork, but Bowker & Zhuang (2015) found no difference in broiler breast muscle with PSE characteristics.
A possible reason could be that myofibrils of broiler breast muscle are less sensitive to PSE-like conditions (van Laack & Lane, 2000).

**ATPase activity**

The most abundant myofibrillar proteins are myosin (43%), actin (22%) and titin (8%). The myosin molecule is the basic unit to form the strand structure of the thick filament. In striated muscles, myosin belongs to the type II family and it is composed by two heavy chains of MW 220 kDa each, two essential light chains (ELC) of MW 17 kDa each, and two regulatory light chains (RLC) of MW 20 kDa each. The total molecular weight of a myosin molecule is thus around 500 kDa.

![Illustration of myosin structure.](image)

As illustrated in Fig. 4, the myosin molecule has two head domains, a neck domain and a tail domain. The N-terminal of each heavy chain forms a globular head domain i.e. the S1 unit, which is in connection to one ELC and one RLC. Each S1 unit contains one ATP-catalytic site and an actin binding site, and this unique structure gives myosin multiple functions (Rayment & Holden, 1994). After the head domain, the two heavy chains start to twist around each other and form a coiled-coil structure. The region adjacent to S1 units is termed the neck domain or the S2 unit. The C-terminal forms the tail domain (rod) i.e. the light meromyosin (LMM). The rods aggregate to form the backbone of the thick filament with S1 units being on the surface of the backbone, and the ones contacting with the thin filament (Miroshnichenko, Balanuk & Nozdrenko, 2000).
Myosin is both an enzyme and a motor molecule. It catalyses ATP, transforming the chemical energy to generate mechanical force. The 3-dimensional structure defines the function of the molecule. Therefore, any alterations occurring in the catalytic domain can result in misrecognition of the substrate and greatly reduce the ATPase activity.

The loss of ATPase activity has been widely used as another indicator of the denaturation of myofibrillar protein, especially in revealing structural alterations within the S1 unit. Offer (1991) established a predicative model of the formation of PSE using the rate constants of myosin denaturation adapted from Penny (1967a). He explained that myosin denaturation can be induced by insufficient pre-rigor chilling, which results in a substantial amount of drip loss. The denaturation is speculated to occur in the head region and therefore results in a loss of ATPase activity (Penny 1967a; 1967b). By incubating myosin at temperature from 32 to 45 °C combined with pH from 5.3 and 6.2, decreases in myosin solubility as well as in ATPase activity were found with increasing temperature and with decreasing pH, indicating the denaturation of myosin at condition with high temperature combined with low pH (Penny, 1976a). In agreement, loss of the myofibrillar ATPase activity has been found in PSE meat compared to normal meat (Sung, Ito & Izumi, 1981; Warner et al., 1997).

One thing need to be noted from literature is that even under conditions similar to PSE, the myosin with an attachment to actin is protected against denaturation (Offer, 1991). Therefore, with rapid glycolysis, a rapid onset of rigor only allows denaturation of a certain portion of myosin. van Laack and Lane (1999) incubated myofibrils from poultry and pork to induce PSE-like denaturation. With additional ATP present for breaking actomyosin bonds, increased loss of solubility was obtained indicating more profound protein denaturation. In agreement, Warner et al. (2014) and Kim et al. (2014) showed that by stretching the sarcomeres during pre-rigor incubation at 37 °C, less ATPase activity was measured at the longer sarcomeres, which also indicates
that reduced overlapping of thick and thin filament resulted in more denaturation of the myosin heads.

In a pre-rigor incubation system (section 2.2.2.), higher temperatures e.g. > 42 °C can induce very rapid glycolysis and thereby the myofibrils experience more severe denaturation conditions than at lower temperatures. However, the time that the myosin heads are susceptible to denaturation is shorter compared to moderate temperatures, because the onset of rigor is much earlier at higher temperatures (Offer, 1991). In a post-rigor incubation system, as rigor is already completed, protection imposed by the attachments of actomyosin against myosin denaturation is at same level among all the temperatures.

Other indicators

Of DSC thermograms from muscles with various water-holding capacities, Peak II (Fig. 3) was the only one significantly correlating with drip loss, which might suggest that denaturation of myosin tails and sarcoplasmic proteins are the ones mainly responsible for the loss of water-holding (Deng et al., 2002). The authors also demonstrated that myosin heads are more sensitive to stress-induced heat denaturation, while the tails and sarcoplasmic proteins are more sensitive to that induced by a low ultimate pH.

In the case of PSE meat, at least 50% of myosin was suggested to denature as indicated by a major decrease in Peak I (Fig. 3) that represents the myosin heads (Stabursvik, Fretheim, & Frøystein, 1984). In line with the studies showing the loss of ATPase activity in PSE meat, these results indicate that the region most susceptible to denaturation under PSE conditions is the myosin head.

Chymotrypsin is a proteolytic enzyme found in pancreatic juice. It cleaves the peptide bonds specifically on amino acids with a side chain that contain an aromatic ring that nicely fits to the “hydrophobic pocket” of the enzyme. If the substrate protein has a compact 3-dimentional molecular structure,
Chymotrypsin will only be able to attack certain hinge regions within a feasible amount of time. When myosin is subjected to chymotrypsin in the presence of EDTA, the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), the light chain i.e. RLC (Fig. 4) is digested. Thereby the myosin S1/S2 junction becomes exposed and is subsequently split up into substructures containing two heads and a rod according to Weeds and Pope (1977) (Fig. 4).

Chymotryptic digestion has been used to detect internal structural changes in the myosin molecule (Takahashi, Takahashi, & Konno, 2005; Yuan, Wang, Chen, Qu & Konno, 2011). Compared to the loss of ATPase activity that mainly reveals the structural alterations occurring in S1 unit, the SDS-PAGE pattern of all the digestion-produced peptides will give additional information regarding the denaturation within the myosin rod. Chymotrypsin digestion technique has been used to investigate the myosin thermal stability in fish muscles. Decreasing rate of HMM (S1 plus S2) production was found, suggesting the loss of native structure due to mild heating in the myosin head region (Takahashi et al., 2005). Myosins extracted from fishes adapted to different temperatures by nature were compared by Yuan et al. (2011). They showed that the structural stability of myosin differed depending on the water temperature that the fish is adapted to. For the same high incubation temperature, myosin from cold-water-adapted fish was more susceptible to chymotrypsin cleavage, which supported the hypothesis that the myosin of a cold-water species is less thermally stable than that of a warm-water species.

Proteins are composed of amino acids with some of their side chains exposed on the surface. The number of hydrophobic side chains determines to what extent the water molecules tend to be pushed by the protein surface. Protein hydrophobicity is one indicator of protein functionality since it reflects the biochemical and structural properties of the protein molecule (Boyer, Joandel, Ouali, & Culioli, 1996). Heating causes the protein structure to become more open and random as driven by entropy, with an increase in the surface
hydrophobicity. The most common way to measure surface hydrophobicity is by fluorescent probes e.g. 1,8-anilino-naphtalene-sulfonate (ANS), cis-parinaric acid (CPA) and 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN). A limitation to using these probes is that they are only applicable to soluble proteins. However, in the case of myofibrils, pre-solubilizing of these structural proteins will create artifacts into the measurements, because the native 3-D structure has already been changed. Bromophenol blue (BPB) has been shown to interact strongly with the hydrophobic sites of protein. Chelh, Gatellier and Santé-Lhoutellier (2006) developed an easy and accurate way to use BPB for determining surface hydrophobicity of myofibrils. By heating from 30 to 70 °C at pH 6.0, myofibrils became increasingly hydrophobic, which was reflected by the amount of BPB bound.

2.3.1.3. Role of denaturation in water-holding

*Influence on lattice spacing*

Based on the evidence as shown above, suggestions have been made in different studies on the mechanism behind of the loss of water-holding in PSE induced by myofibrillar protein denaturation. Offer and Knight (1988b) proposed a hypothesis that the denaturation of myosin shrinks the myofibrils laterally, and therefore expelling the water to the extracellular space. As explained in 2.1.3.1 steric forces greatly influence the water-holding of meat since the volume of the sarcomere defines the amount of water that can be held within it. When rigor is completed, thick and thin filaments are locked up together. The length of S1 + S2 units has impact on the lattice spacing. Goldman et al. (1979) showed that at the same sarcomere length, the attachment of cross-bridges (S1+S2) shrank the lattice spacing by around 12% and the cross-bridges kept the lattice spacing at an optimum length. At other spacings, the lateral force tends to displace the filament lattice toward that optimum value (Matsubara, Goldman & Simmons, 1984). The results of reduced ATPase activity in PSE meat can be interpreted as an indication of
structural alterations of myosin specifically in the S1 and S2 region. Therefore, it is possible that this conformational change will also result in a reduction in the length of head, thus pulling the neighbouring filaments closer to each other. The additional shrinkage of myofibrils as whole will expel the water to the extracellular space. At the same time the shrinkage enlarges the extracellular space where water is held less firmly and is thus susceptible to be lost afterwards. This has been supported by microscopy examination of the microstructure of the fibres. Larger gaps between fibres were thus observed in PSE compared to normal meat (Barbut, Zhang & Marcone, 2005).

Electron microscopy was used to measure shortening of the myosin molecule in a solution upon heat denaturation in a temperature interval of 20 to 50 °C. At a temperature around 40 °C, the myosin molecule was reduced by 20 nm in length and most of the shrinkage took place in the region between S2 and LMM (Walker & Trinick, 1986). Since S2 is part of the cross-bridge structure connecting thick and thin filaments in rigor, this shrinkage may also occur when myosin is in filamental structure, and might thereby reduce the filamental spacing. Electron microscopy requires fixing, staining and drying the specimen before examination, which is likely to induce shrinkage of the lattice spacing yet as an artefact. An alternative way to measure the lattice spacing is by low angle X-ray diffraction. This technique has been used to determine the microstructure in muscle physiology studies and later on in meat science studies e.g. to measure the lateral spacing between filaments during rigor formation and thereby increasing the understanding of the structural changes accounting for drip loss (Diesbourg et al., 1988), and the relationship between lattice spacing and sarcomere length (Schäfer et al., 2000). More recently, a related technique, small angle X-ray scattering (SAXS) was shown to be a powerful research tool to determine intramuscular fat (IMF) content in lamb, an information that was obtained additional to lattice spacing (Hoban et al., 2016). The advantage of X-ray diffraction is that it can be directly applied to a wet sample without requirement of any extra pre-
treatments, and therefore the results better reflect the original condition in the muscle.

![Diagram of X-ray diffraction apparatus](image)

**Fig. 5.** A: Schematic illustration of the low angle X-ray diffraction apparatus. B: Diagram of the filament lattice of in the overlap region of the sarcomere. Thick filaments are represented by solid dots and with the outer circle representing the “cross bridge” region. Thin filaments are presented by smaller solid dots. C: Diagram of the first 8 orders of the equatorial diffraction pattern from the hexagonal A-band lattice (Millman, 1998).

In the low angle X-ray diffraction measurement, the X-ray beam is passed through a muscle sample (0.3-1 mm thick) along the transversal axis of the muscle fibre. The diffraction pattern will be observed and collected on a detector, which is placed at a suitable distance away from the sample. From the fibrous structure, usually two axes of patterns will be observed. The axis that is parallel with fibre direction is referred to as the meridian and the perpendicular axis as the equator (Fig. 5A). Diffraction patterns that lie along the equator are termed the equatorial reflections and they originate specifically from the lattice structure in the A-band (Fig. 5B). In the vertebrate striated muscle, most of the intensity is in the reflections 1,0 and 1,1. These reflections arises from the lattice planes in the hexagonal unit cell as shown in Fig. 5B. By measuring the separation of these reflections from the centre of the X-ray pattern (S) and by entering it into the Bragg’s equation, the spacing of $d_{1,0}$ and $d_{1,1}$ in the lattice structure can be calculated as:
\[ d_{1,0}(d_{1,1}) = \frac{2\lambda\delta}{S_{1,0}(S_{1,1})} \]

\(\lambda\) is the wavelength of the X-radiation and \(\delta\) is the distance from the sample to the detector.

As stated by Offer and Knight (1988), the lattice spacing within PSE meat at 24 h post mortem was reduced to 50% of the values measured in the normal meat by X-ray diffraction. In agreement, Irving, Swatland and Millman (1989) showed smaller lattice spacing in PSE than in DFD (39 vs 46 nm). Although the sarcomeres were longer in PSE meat, the calculated lattice volume (based on a simplification as a cylinder) was reduced by nearly 40% in comparison to DFD. As a consequence to the additional lateral shrinkage within PSE fibres, the proportion of extracellular space was enlarged (Swatland, Irving & Millman, 1989).

However, even if a substantial shrinkage on of the lattice spacing can be directly (by X-ray diffraction) or indirectly (by the loss ATPase activity) shown in PSE meat, those being suggestive of the role of myosin denaturation within the loss of water-holding, it is difficult to conclude that myosin denaturation is an absolute causative factor. This is mainly because simultaneously with that of myosin denaturation is occurring also the denaturation of sarcoplasmic proteins that adds to the effects observed in the water-holding, as will be further described in sections 2.3.2 and 2.3.3.

**Proteolytic degradation**

The longitudinal and lateral post mortem shrinkage of myofibrils triggers shrinkage of the muscle fibre volume, creating gaps between the fibres and subsequently enabling the formation of drip channels, as previously discussed in section 2.1.4. The shrinkage force will not, however, be transmitted from myofibrils into the entire muscle fibre without the physical connection between myofibrils to the sarcolemma. This connection is accommodated by the intermediate filaments as part of the cytoskeleton.
involving proteins like desmin, filamin and vinculin. Desmin is a substrate for the calpains (Baron, Jacobsen & Purslow, 2004), a group of proteolytic enzymes that are activated post mortem (Koohmaraie, 1992). Calpains belong to the family of cysteine proteases with members such as the \(\mu\)-calpain, m-calpain, and p94 (or calpain 3). \(\mu\)-Calpain and m-calpain are named by the required concentration level of \(\text{Ca}^{2+}\) for enzyme activation. In meat science, the proteolytic activity of calpains has been intensively studied in relation to meat tenderness (Kemp, Sensky, Bardsley, Buttery & Parr, 2010) and has also been suggested to be involved in water-holding (Huff-Lonergan & Lonergan, 2005). A hypothesis regarding the increase in water-holding during aging was proposed by Kristensen and Purslow (2001) and is shown in Fig. 6. During aging, the cytoskeleton is gradually degraded by the proteolytic enzymes, and the linkages between myofibrils and sarcolemma loosen up. As a result, the extracellular water that has been pressed out to the extracellular space will flow back into the muscle fibre and this is believed to be one explanation for the increased water-holding observed with proceeding proteolytic degradation (Huff-Lonergan & Lonergan, 2005; Melody et al., 2004; Morrison, Mielche & Purslow, 1998; Zhang, Lonergan, Gardner & Huff-Lonergan, 2006).
A question arises: is the proteolytic activity affected also in the case of PSE? Although PSE meat is described as “soft”, its proteolytic characteristics, especially with respect to water-holding, have not been much studied. Some suggestions can, however, be found in studies concerning the influence of pre-rigor conditions on water compartmentalization and tenderization. With NMR, Tornberg et al. (2000) observed an enlarged extracellular space and a subsequent early outflow of sarcoplasm along with significant tenderization to occur in beef with fast pH decline post mortem. The result is in agreement with that of Dransfield (1994), who showed that rapid pH decline induced calpains to become activated earlier than is usually seen. Bee, Anderson, Lonergan and Huff-Lonergan (2007) found that a rapid early post mortem pH decline gave rise to the activation of μ-calpain, which might partly account for the increased drip loss. The intracellular concentration of Ca^{2+} increases due to high temperature, which can induce the autolysis of native calpains into active forms (Pomponio & Ertbjerg, 2012). However, the early activation of calpains may be only limited to a short time as stated by Dransfield (1994).
and later supported by Warner et al. (1997) showing less degraded titin in PSE compared to normal meat. Therefore, it can be speculated that should the development of PSE characteristics be due to high pre-rigor temperatures, could this elevated temperature also result in early activation of calpains as a side-effect?

Fig. 7. Schematic representation the cytoskeletal structures and proteins susceptible to post-mortem cleavage (Taylor et al., 1995).

Titin is responsible for anchoring thick filaments to Z-lines and it is slowly degraded by proteolytic enzymes such as the calpains during aging (Takahashi & Saito, 1979). Controversial to the previous discussion about rapid pH drop resulting in accelerated proteolysis, less degradation of titin was observed in samples with an abnormally rapid glycolysis induced by PSS (Boles et al., 1992). The authors argued that the lower level of degraded titin might be attributable to an alteration within its molecular structure, making it less susceptible to proteolysis. Based on the myofibrillar location of titin, the current author is inclined to believe that titin’s degradation might be more of a
factor accounting for the loss of the extractability of myofibrils than one responsible as such for the loss of water-holding in PSE meat.

### 2.3.2. Sarcoplasmic protein denaturation

#### 2.3.2.1. Gelation upon heating

Sarcoplasmic proteins are a group of various globular proteins. Contrary to fibrous proteins, the globular proteins tend to expand and unfold upon heating. Unfolded proteins can continue to aggregate with neighbouring proteins, and thereby form a heat-induced gel. This process involves three steps: initial protein unfolding, linear fibrillar aggregation, and random cross-linking between fibrils (Clark, Kavanagh & Ross-Murphy, 2001). The behaviour of denatured globular proteins in each step as well as the progress thereafter are both greatly dependent on factors like temperature, time, net charges of the proteins that is related to how far the medium pH is away from proteins’ pI, and ionic strength. In meat system, apart from globular sarcoplasmic proteins there are also other components. Thus protein gelation is additionally influenced by the insoluble compounds e.g. meat fibres, connective tissue and fat cells (Tornberg, 2005). Due to their capacity in gel formation, the heat-coagulated sarcoplasmic proteins are believed to contribute to the rigid consistency of cooked meat products since their gelling may act as a binder between the structural elements such as myosin and actin filaments in meat. In production of Surimi gel, a thermally treated gel mainly formed by extracted fish myofibrillar proteins, adding sarcoplasmic proteins during manufacturing has been shown by many studies to enhance the gel strength as reviewed by Jafarpour and Gorczyca (2012).

DSC thermograms show that sarcoplasmic proteins give an endothermic peak at around 67 °C (Fig. 2.). Severe precipitation of sarcoplasmic proteins starts when the temperature is above 40 °C (Hamm & Deatherage, 1960; Miyaguchi, Nagayama & Tsutsumi, 2000). Only 40% of the proteins remained soluble at 60 °C and it dropped down to less than 1% at 80 °C at pH 6.5 (Miyaguchi et al.,
Sarcoplasmic proteins have pI above 5.5 and the onset of precipitation can start at even lower temperatures if pH shifts from neutral to lower values. When pH is at 5.5, incubation at 37 °C and 45 °C result in substantial precipitation of sarcoplasmic proteins, 15% and 35% respectively (Scopes, 1964). Heat-set gel formed solely by denatured sarcoplasmic proteins (concentration 5%) after 30 min at 80 °C was able to hold 40% of water after centrifugation 1000 g x 20 min. Miyaguchi, Hayashi and Sakamoto (2007) used electron microscopy to demonstrate that a network was formed between myofibrils and sarcoplasmic proteins upon heating at 70 °C for 30 min. This protein network appeared condensed and fine as a gel and denatured sarcoplasmic proteins built up porous structures between denatured myofilaments, resulting in a gel matrix good at holding water.

The PSE-like condition involves temperatures in the range up to around 44 °C, and therefore the denaturation of sarcoplasmic proteins will not be as severe as that occurring in heating as discussed above. However, heat-set gel formation and its contribution to the functionality of meat product can still shed light to the manner of denaturation of sarcoplasmic proteins in the PSE condition.

2.3.2.2. Evidence in PSE

The sarcoplasmic protein fraction can be obtained by homogenization of meat in a buffer with low ionic strength, centrifugation and collecting of the subsequent supernatant. By measuring the protein content in the supernatant, the solubility of sarcoplasmic proteins can be known, which has been shown to be significantly lower in PSE meat by numerous studies. Therefore, the loss of solubility has been widely used as an indicator of the denaturation occurring in PSE meat (Joo et al., 1999; Maribo et al., 1998; Warner et al., 1997; Zhu et al., 2011).
The reduction in the total amount of extracted sarcoplasmic proteins is caused by the precipitation of some specific enzymes. The two most studied enzymes are glycogen phosphorylase and creatine kinase.

Glycogen phosphorylase is approximately 4.5% of the total sarcoplasmic proteins within muscle (Scopes, 1970). It acts as a critical enzyme for energy supply by deducting glucose from glycogen during glycogenolysis (Melendez-Hevia, Waddell & Shelton, 1993). Glycogen phosphorylase is a dimer and the MW of each monomer is 97 kDa (Johnson, 1992). Phosphorylase is not associated with any structural proteins within myofibrils in normal muscle (Pietrzak et al, 1997). However, phosphorylase may be associated with sarcoplasmic reticulum (Cuenda, Nogues, Henao & Gutiérrez-Merino, 1995) via binding to the glycogen particles (Wanson & Drochmans, 1972).

Creatine kinase constitutes about 9% of the total sarcoplasmic proteins (Scopes, 1970). It is responsible for converting creatine with ATP to produce phosphocreatine in cells. This is critical in skeletal muscle and brain where phosphocreatine can be used as a rapid energy supply to regenerate ATP (Wallimann, Wyss, Brdiczka, Nocolay & Eppenberger, 1992). Creatine kinase in skeletal muscle is mainly composed by M-subunits with MW 43 kDa (Perryman, Struss, Buettner & Roberts, 1983). By immunofluorescent blotting, part of the creatine kinase was located on the M-line of myofibrils (Turner, Wallimann & Eppenberger, 1973), which takes 3-5% of the total creatine kinase present within muscle fibre (Wallimann, Turner & Eppenberger, 1973).

Except a small part that is associated with myofibrillar structure, most of the enzymes in sarcoplasm are soluble and relatively freely present, and some are susceptible to the PSE denaturation condition. Warner et al. (1997) showed that phosphorylase and creatine kinase became insoluble in PSE, and later Joo et al. (1999) also documented that these two enzymes precipitated from the sarcoplasmic protein fraction in PSE and appeared in the myofibrillar
proteins in pork. Bowker and Zhuang (2015) confirmed this by Western blot in poultry. In these techniques, the structures of the proteins are destroyed and the results only tell the specific sarcoplasmic proteins that translocate from the soluble to the insoluble protein fraction. Pietrzak et al. (1997) used primary and secondary antibodies to blot myofibrils with its original structure and then observed the fluorescent signal under microscope. Under high temperature and low pH condition, fluorescent signal from phosphorylase was found specifically on the Z-lines as well as in the middle of A-bands in turkey samples. This indicates that phosphorylase does not precipitate randomly, but more likely in a way associated with structural elements. However, the mechanism behind is unclear. Drip originate from sarcoplasm, and Savage et al. (1990) and den Hertog-Meischke, van Logtestijn and van Knapen (1996) showed corresponding decreased amount of phosphorylase and creatine kinase in the drip collected from meat with poorer water-holding, indicating that these enzymes become insoluble.

BisANS (4, 4'-dianilino-1, 1'-binaphthyl-5, 5'-disulfonic acid) is able to bind to the hydrophobic sites on protein molecules and it has been used to study the conformational changes of sarcoplasmic proteins caused by pH and mild heating. Creatine kinase was shown to become less hydrophobic when pH dropped from 7.5 to 5.5 while glycogen phosphorylase was not influenced. No difference was observed between temperatures below 40 °C, indicating that sarcoplasmic proteins will only start to denature when the incubation temperature is around 40 °C will it start to denature sarcoplasmic proteins (Gratacós-Cubarsí & Lametsch, 2008).

Pre-rigor incubation of muscle at elevated temperature is able to induce sarcoplasmic protein precipitation accompanied with substantially high drip loss (Zhu et al., 2011). Fischer at al. (1979) was the first to prove that post-rigor temperature incubation at 40 °C can simulate similar denaturation in glycogen phosphorylase and creatine kinase compared to such in PSE meat.
2.3.2.3. Role of denaturation in water-holding

Bendall and Wismer-Pederson (1962) were the first to propose that sarcoplasmic protein denaturation is the causative factor for the loss of water-holding capacity in PSE. They argued that the sediment of precipitated sarcoplasmic proteins on the surface of myofibrils will act as barrier for myofibrillar protein to interact with water. This layer of denatured sarcoplasmic proteins rather than the structural changes within the myofibril itself causes the loss of functionality of myofibrils such as reduced extractability. This interpretation is supported by the observation that myofibrils from PSE meat had the same pI as normal meat even the overall water-holding capacity level was lower. In contrast, for heat-aggregated myofibrils at 90 °C, the pI was shifted to a higher value (Bendall & Wismer-Pederson, 1962). These authors argued that the denaturation occurred in myofibrils is in a different manner compared to high temperature induced denaturation, and thus the denatured sarcoplasmic proteins are more likely to be responsible for the altered functionality of myofibrils. Hamm and Deatherage (1960) showed the influence of pH on the water-holding of the mild-heated meat (40 °C), supporting the results of Bendall and Wismer-Pederson (1962) that after heating, the pI is not changed compared to fresh meat.

Lopez-Bote, Warriss and Brown (1989) and later Joo et al. (1999) also observed a correlation between water-holding and the solubility of sarcoplasmic proteins in pork, and the correlation was higher than with the solubility of myofibrillar proteins. This observation has been cited in many studies as an argument that it is sarcoplasmic protein denaturation that causes the loss of water-holding in PSE meat. More recently Bowker and Zhuang (2015) also found a significant correlation between the sarcoplasmic protein solubility and water-holding, but not to the myofibrillar protein solubility. It may be explained by the differences between animal species, that myofibrils from poultry are more resistant to denaturation compared to myofibrils from pork.
(van Laack & Lane, 2000). However, from the current author’s opinion, a good correlation coefficient of water-holding with sarcoplasmic protein solubility only can show the co-occurrence of these two factors, but not necessarily the causative relationship between them. To be able to conclude that this correlation reflects a direct relationship, more solid evidence revealing the precise mechanism still remains to be shown.

The most known hypothesis explaining how precipitated sarcoplasmic proteins can diminish the water-holding is called “shielding of charges”. As suggested by Bendall and Wismer-Pedersen (1962) and later supported by other papers (Boles et al. 1992; Zhu et al., 2011), the sediment of denatured sarcoplasmic proteins shields the charged groups on the surface of myofibrils, and thereby interfering the interaction between charged side chains with water molecules.

However, first Monin and Laborde (1985) and later on Hertog-Meischke et al. (1996) and Wilson and van Laack (1999), were skeptical about the above hypothesis. First of all, sarcoplasmic proteins have been estimated to only account for binding 3% of the total water in meat (Hamm, 1972) and therefore it is not likely the explanation of the big difference in water-holding between PSE and normal meat. In addition, as described in 2.3.2.1, globular proteins tend to unfold and form a gel upon denaturation, which should in turns trap more water.

In fact an extract of sarcoplasm was observed to have a positive effect on the water-holding capacity of myofibrils without any heat denaturation (Monin & Laborde, 1985), although in this study, an extract was used and therefore the influence from non-protein constitutes cannot be excluded. Hamm (1962) showed a clear positive effect of the non-protein small molecules on the water-holding. Sarcoplasm extracted from normal meat also had a positive impact on the water-holding of myofibrils compared to an extract from PSE meat. No difference was found in ionic strength, therefore, it was speculated
that the presence of sarcoplasmic proteins in sarcoplasm contributes to the osmotic value, thus pulling water into the filaments (Wilson and van Laack, 1999).

2.3.3. The interaction between sarcoplasmic and myofibrillar proteins

The denaturation of myofibrillar and sarcoplasmic proteins co-exist during the development of PSE meat. The poor water-holding capacity observed later on is rather an accumulative result of the denaturation occurring in both myofibrillar and sarcoplasmic proteins. Therefore, it is very hard to figure out the causative relationship between either one of them with the loss of water-holding capacity in PSE.

Selecting the indicators of myofibrillar and sarcoplasmic protein denaturation is very important. For instance, for the solubility of myofibrillar proteins, Warner et al. (1997) argued that phosphorylase becomes irreversible bound to myofibrils and possibly prevent the process of myosin tail unravelling in the extraction process, but it is not clear whether the reduction in the solubility of myofibrillar proteins is caused by the structural changes in myofibrils or the sediment layer of precipitated sarcoplasmic proteins. The same argument applies for reduced ATPase activity and increased surface hydrophobicity measured from PSE myofibrils. When these indicators are measured, the precipitated sarcoplasmic proteins are already bound to the surface of myofilaments. Therefore, to understand the subsequent results, the impact from denatured sarcoplasmic protein cannot be neglected. We can even imagine a scenario where the sarcoplasmic protein precipitation is induced in the first place by the PSE condition, and the sediment of these denatured proteins thereafter causes the reduced extractability (solubility) of myofibrillar proteins.

To better understand the independent role of the sarcoplasmic and myofibrillar protein denaturation on the water-holding capacity in PSE meat, these two protein fractions need to be separated before the denaturation
condition is applied. Thereby, the impacts from each of them can be analysed independently and compared.

3. Objectives

The aim of the thesis is to understand the role of sarcoplasmic protein and myofibrillar protein denaturation regarding their impacts on the water-holding capacity of meat. To achieve this, pre-rigor and post-rigor incubations at relatively high temperatures were applied in order to induce PSE-like protein denaturation to muscle, muscle fibres and myofibrils. The specific objectives are:

- To investigate the effect of pre-rigor temperature incubation on the sarcoplasmic protein characteristics in relation to meat properties within porcine LTL muscle (paper I)
- To investigate the effect of pre-rigor temperature incubation of porcine LTL muscle on the myofibrillar protein characteristics and especially in relation to myosin properties (paper II)
- To compare the independent influences of denatured sarcoplasmic and denatured myofibrillar proteins, as well as the interaction between them on the water-holding of myofibrils (paper III)
4. Materials and Methods

General description of the materials and methods used in the current thesis are summarized here. More details can be seen in the publications attached (I, II, III).

4.1. Sampling and temperature incubation

Muscle

LTL muscles were used throughout the whole thesis. They were from female pigs from the crosses Norwegian Landrace × Swedish Yorkshire × Danish Landrace. Six muscles were obtained from four carcasses for the re-rigor incubation in the slaughter house in Study I & II. Samples from six muscles were used for meat quality analysis and sarcoplasmic protein analysis in Study I. Samples from three muscles were used in Study II. Ten muscles were obtained from ten carcasses in Study III.

Pre-rigor incubation

In Study I and II, pre-rigor incubation was done at 45 min post mortem at temperatures 0, 10, 20, 30 and 40 °C until 6 h post mortem.

Post-rigor incubation

In Study III, in the first experiment myofibrils and sarcoplasm were extracted from 24 h post mortem muscles and then mixed for incubation at temperatures from 21 to 44 °C for 1h. Details are described in 2.5.1. (Study III). In the second experiment, myofibrils were incubated with different amount of sarcoplasmic proteins and subjected to incubation only at 44 °C for 1h. Details are described in 2.6. (Study III). In the last part, for the of X-ray diffraction measurement, fibre bundles were dissected from 24 h post mortem muscle and then subjected to incubation at 21 and 44 °C with sarcoplasm/protein-depleted sarcoplasm. Details are described in 2.7.2. (Study III).
4.2. Protein fraction preparation

In Study I, muscle samples for protein analysis were taken at 6, 24, 48 and 72 h post mortem from temperature groups 0, 10, 20, 30 and 40 °C. In Study II, muscle samples were taken at 24 h post mortem and from temperature groups 20, 30 and 40 °C. In Study III, muscles at 24 h post mortem were used to extract myofibrils and collect sarcoplasm.

Sarcoplasmic proteins and myofibrils were first separated in rigor buffer by homogenization and centrifugation. After that myofibrils were washed one more time in rigor buffer as described in Study I. Same procedure was done in Study II with only difference in using MOPS as buffering reagent in rigor buffer and two times of washing. After washing, the final pellet containing mainly myofibrils was resupended in rigor buffer (Study I), or in digestion buffer (Study II). In Study III, after the first homogenization in Tris rigor buffer and subsequent centrifugation, myofibrils were washed twice in a buffer with pH 5.5 and left as pellet for the use as myofibrils.

Drip was used as sarcoplasm in Study III. A meat piece (~ 80 g) was cut across the length of LTL muscle (~ 1.4 cm x muscle diameter), and several cuts were made on the surface penetrating a few mm towards the center to facilitate the migration of drip. The meat piece was then sealed in a polyethylene bag. Drip was collected from the bottom of the bags after hanging meat pieces from 24-48 h post mortem at 4 °C. After 80 °C denaturation and subsequent centrifugation, the supernatant was used as protein-depleted sarcoplasm in Study III. The protein profile was checked and compared with sarcoplasmic protein fraction by SDS-PAGE (Fig. 1, Study III).

In Study III, the pellet after homogenization of meat and subsequent washing steps was used as myofibrils. After 10,000 g of centrifugation, the pellet still contained buffer apart from proteins (myofibrils). The ratio of 1:1 (v:w) was
the sarcoplasm (drip) : myofibrils ratio during the mixing step. The ratio of 1:1.3 was the sarcoplasmic : myofibrillar protein content ratio in the mixture.

4.3. Physical analysis

Pre-rigor pH was measured by stopping the glycolysis by homogenizing 0.4-0.5 g of meat in 10 volumes of buffer (5 mM Na-Iodoacetic acid, 150 mM KCl) and then a determination by a pH electrode (Study I). Post-rigor pH was measured by directly inserting a pH electrode into the tissue (Study III).

The gravimetric method (Honikel, 1998) was used for drip loss measurement of muscle pieces in Study I and III. A meat piece (~ 80 g) was cut across the length of LTL muscle (~ 1.4 cm x muscle diameter), weighted and sealed in a polyethylene bag. It should be noted that these meat samples were not the same as the ones for collecting drip for the use of sarcoplasm in the incubation. The bag was hanging by penetrating the meat with thread, and ensuring that meat did not touch any side of the bag. Hanging was at 4 °C until desired storage time was reached. Then the bag was opened and the exudative on the surface was removed carefully by tissue paper. The meat was weighted again. The weight difference expressed as a percentage of initial weigh was used as drip loss. The centrifugation method as described in Study III was used for water-loss measurement of myofibrils.

Cooking loss and tenderness were measured and described in Study I at 72 h post mortem. Shear force was analysed by the Allo-Kramer method (Zhu et al., 2011).

The sarcomere length was measured in Study I by the light diffraction method according to Cross, West and Dutson (1981) at 2, 4, 6 and 24 h post mortem. A piece of meat was finely minced and homogenized by a Potter-Elvehjem homogenizer for 15 s in a 3.5 % formaldehyde solution and a few drops of sample were added onto a microscope slide. The glass slide with the meat homogenate was moved slowly under the laser beam until the beam passed through a line of sarcomeres and a red diffraction pattern was
displayed. The distance from the center to one of the sides of the pattern (T in mm) was measured. The distance from the slide holder and the screen (D in mm) was also measured. Then sarcomere length was determined by the following equation:

\[
\text{Sarcomere length (μm)} = \frac{0.6328 \times D \times \sqrt{T^2 + 1}}{T}
\]

Low angle X-ray diffraction was used to determine the lattice spacing within sarcomeres according to Li, Andersson-Lendahl, Sejersen and Arner (2013) in Study III. The set-up of the apparatus is described in 2.7.3. (Study III)

4.4. Biochemical analysis

Protein determination

RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) was used throughout the whole thesis. Myofibrils were first solubilized by adding 1% of SDS prior to the determination.

SDS-PAGE and Western blot

SDS-PAGE and Western blot were done in Study I and II. Electrophoresis apparatus from Invitrogen XCell Sure Lock® were used. NuPAGE® 7% Tris-Acetate gels (Invitrogen) were used in Study I, 12% Bis-Tris gels (Invitrogen) were used in Study II. The protocol of SDS-PAGE and Western blot including samples preparation, running condition, staining and quantification is described in 2.4.1 and 2.4.2, Study I.

Ca²⁺ ATPase assay

The principle of the assay is to measure the liberated inorganic phosphate by ATPase from either myofibrils or S1 unit, during a certain period of time. The assay was first run in Study II according to Silva, Sparrow and Geeves (2003) with slight modifications. In Study III, same principle was followed with
further modifications to simplify the procedure. The assay was carried out in micro plate and PCA addition step was omitted.

*Surface hydrophobicity*

Surface hydrophobicity of myofibrils was measured according to Chelh et al. (2006), and the protocol was described in 2.3, Study II.

*Calpain zymography*

In Study I calpain activity was measured by using casein zymographic gels according to Pomponio et al. (2008). Calpains from meat at 50 min, 6, 24, 48 and 72 h post mortem from all the incubation temperatures were extracted according to the protocol described in 2.4.4, Study I.

*Chymotrypsin cleavage*

Chymotrypsin cleavage was used in Study II to cleave the S1/S2 junction in the myosin molecule. Since the S1 unit is a soluble protein, a fraction containing the S1 unit was separated from the remaining filamental structures by centrifugation. The procedure was described in Study II.

4.5. Statistical analysis

Analysis of variance (ANOVA) was done by JMP® 9.0.0 (JMP, Cary, NC) using the Fit Model platform in all the studies. Animal number was defined as a random factor. Least square means were compared by Tukey’s HSD (honest significant difference) test using the pairwise comparison method.
5. Summary of results

5.1. Effect of pre-rigor incubation on meat quality

Pre-rigor incubation at temperatures from 0 to 40 °C for 6 h altered the pH decline successfully and muscles incubated at 40 °C had the fastest pH drop (Fig. 1, Study I). Meat that experienced relatively high temperature and low pH developed the PSE-like characteristics such as significantly higher drip loss at 24, 48, and 72 h post mortem ($P < 0.001$). Sarcomere lengths were the same at 24 h post mortem ($P > 0.05$). Substantial tenderization was found in meat incubated at 40 °C ($P < 0.01$) (Table 1, Study I).

5.2. Effect of pre-rigor incubation on protein characteristics

*Sarcoplasmic proteins*

Pre-rigor temperature incubation lowered the protein content in the sarcoplasmic fraction ($P < 0.001$) (Table 2, Study I). Simultaneously an increase ($P < 0.001$) of protein content in the myofibrillar fraction was observed, indicating the precipitation of sarcoplasmic proteins. It was confirmed by SDS-PAGE and Western Blot that glycogen phosphorylase and creatine kinase had translocated from the sarcoplasm to the myofibrils due to the incubation at 40 °C (Fig. 2 and 3, Study I). No impact from storage time of 6 to 72 h post mortem was found on the amount of precipitated sarcoplasmic protein ($P > 0.05$) (Fig. 4, Study I).

Differences in the calpain activity were found between different incubation temperatures. After incubation at 40 °C for 6 h, the activity of extractable $\mu$- and m-calpains decreased dramatically compared to the lower temperatures ($P < 0.001$). During following storage from 6 to 72 h post mortem, calpain activity measured from temperature groups lower than 40 °C continued to drop (Fig. 5 and 6, Study I).
Myofibrillar proteins

At the myofibrillar level, not only the protein content was increased after incubation at 40 °C, but also the surface hydrophobicity was higher compared to lower temperatures \((P < 0.001)\) (Fig. 1, Study II). Ca\(^{2+}\) ATPase activity was reduced dramatically by incubation at 40 °C \((P < 0.001)\) (Fig. 2, Study II).

At the myosin level, chymotrypsin cleaved myosin molecules that had been incubated at 40 °C in a different manner compared to 20 and 30 °C. Differences were found in the protein content of different fractions obtained after chymotryptic digestion and subsequent centrifugations (Table 1, Study II). In addition, the protein profiles in all the fractions were shown by SDS-PAGE, and the most obvious difference was that no intact S1 units were obtained after the digestion (Fig. 3, Study II). Reduced Ca\(^{2+}\) ATPase activities were found in all the fractions following incubation at 40 °C \((P < 0.05)\) (Fig. 2, Study II). Phosphorylase and creatine kinase were completely digested after the chymotryptic digestion (Fig. 4, Study II).

5.3. Effect of denatured sarcoplasm on water-holding

The protein concentration in drip for the use as sarcoplasm during incubation was measured in drip collected from 6 different LTL muscles in Study III. The results showed that the drip contained \(148 \pm 13\) mg/ml of proteins.

In Study III, experiment 2.4, the influence from denatured sarcoplasm and myofibrillar proteins were studied and shown in Fig. 2A and 2B. The different experimental lines were designed as following:

Green triangle: myofibrils were incubated alone. In this group, only myofibrils experienced post-rigor incubation at different temperature. At temperatures higher than 38 °C, denaturation started to occur in the myofibrillar proteins. Before centrifugation for water-holding determination, unheated sarcoplasm was added back to all the temperature groups in order to bring back the same
amount of water and soluble proteins as the samples in the other experimental lines. This group was set up as a control group.

**Red squares:** myofibrils and sarcoplasm were incubated separately and thereafter mixed together. Both myofibrils and sarcoplasm experienced temperature-induced denaturation, however, without interaction between two protein fractions during the denaturation. Since the sarcoplasmic proteins coagulated in an independent system, they had no possibility to precipitate onto the filaments.

**Blue dots:** myofibrils and sarcoplasm were mixed before the incubation. This group is the closest to the situation in the native muscle. Myofibrils and sarcoplasm were incubated in one system and, therefore, when denaturation started to proceed, there was an interaction between the denatured soluble and structural proteins.

Post-rigor incubations from 21 to 44 °C had significant impact on the water-holding of myofibrils (P < 0.001) (Table 1, Study III). As shown in Fig. 2A, Study III, denatured myofibrils without denatured sarcoplasm had the highest water-loss ([MF], Green triangles). Denatured myofibrils after adding denatured sarcoplasm had the intermediate water-loss ([MF]+[SP], Red squares) and the myofibrils denatured together with sarcoplasm had the best water-holding capacity (P < 0.001) ([MF+SP], Blue dots).

The surface hydrophobicity of myofibrils increased upon elevated incubation temperature among all the three treatments groups (P < 0.001) (Fig. 2B, Study III). However, no significant difference was found between the treatment groups (Table 1, Study III).

**5.4. Effect of denatured sarcoplasmic proteins on water-holding**

The concentrations of the sarcoplasmic proteins in the sarcoplasm were adjusted by adding protein-depleted sarcoplasm. The treatments for groups represented by Green Triangle and Blue Dots in Fig. 3. (Study III) were similar
as described for Fig. 2A and Fig. 2B above, with the difference that only 44 °C was applied with different sarcoplasmic protein concentrations. Water-loss decreased dramatically with an increasing amount of sarcoplasmic proteins in the sarcoplasm, both in their native and denatured state \( (P < 0.001) \) (Fig. 3, Study III).

5.5. Effect of denatured sarcoplasmic proteins on lattice spacing

Fibre bundles were used in the X-ray diffraction measurement to investigate the effect of denatured sarcoplasmic protein on the distances between the thick and thin filaments. Fibre bundles were first treated with detergent in order to make the sarcolemma permeable and to remove the sarcoplasm, a process which is called “skinning”. Fig. 4A, Study III demonstrated that skinning was efficient to remove the sarcoplasm from fibres that have not been temperature incubated, and that it was possible to bring back the sarcoplasm and protein-depleted sarcoplasm to the fibre bundles later on.

Values of \( d_{1,0} \) and \( d_{1,1} \) reflected the lateral distances between the filaments in the sarcomere. As shown by Fig. 4B (Study III), post-rigor incubation at 44 °C shrunk the lattice spacing indicated by \( d_{1,0} \) and \( d_{1,1} \) regardless of the presence of sarcoplasmic proteins \( (P < 0.001) \). In addition to the shrinkage of lattice spacing due to the myofibrillar protein denaturation at 44 °C (Black bars, 21 vs. 44 °C), the presence of denatured sarcoplasmic proteins is associated with even more compression in the lattice spacing \( (P < 0.001) \) (Gray vs. Black bars, 44 °C).
6. General discussion

6.1. Protein denaturation accompanied with loss of water-holding

*Water-holding is reduced by pre-rigor/post-rigor temperature incubation*

In Study I and II, pre-rigor incubation was applied to the muscle samples up to 6 h post mortem. The incubation successfully accelerated the pH decline when the temperature was above 30 °C (Fig. 1. Study I). The decline rates were 0.10 unit/h at 0 to 20 °C, 0.24 unit/h at 30 °C and 0.3 unit/h at 40 °C approximately. This is in agreement with Klont et al. (1994), Maribo et al. (1998) and Zhu et al. (2011) that early post mortem high temperature induced a rapid pH decline as in PSE meat. However, the fastest pH decline obtained in Study I was slower compared to moderate PSE meat (Bendall & Swatland, 1988; Offer, 1991).

PSE-like characteristics were found in the meat after incubation at 40 °C i.e. significantly higher drip loss, which was 12% at 24 h post mortem (Table 1. Study I). This is in the range of drip losses observed from mild to extreme PSE case (8.6-11.6%) as described by Joo et al. (1999). Although 30 °C resulted in numerical higher drip loss than at the lower temperatures, the difference was not significant (P > 0.05). Therefore, the critical temperature point for protein denaturation seems to be somewhere between 30 and 40 °C, which is in agreement with literature that pre-rigor temperature incubation needs to be higher than 37 °C in order to decrease the water-holding significantly as summarized in a review by Kim et al. (2014).

In Study III, in the first two experiments (2.5 and 2.6) the experimental subject was switched to myofibrils in rigor. Post-rigor temperature incubation of myofibrils (pH 5.6) affected water-loss significantly (Table 1. Study III), which is in line with Monin and Laborde (1985). In agreement with studies from Monin and Laborde (1985) and van Laack and Lane (2000), Study III showed that temperatures higher than 38 °C is able to induce PSE-like characteristics.
Sarcoplasmic protein precipitation

In Study I, pre-rigor incubation at 40 °C greatly reduced the total amount of proteins in the sarcoplasmic fraction. As illustrated by SDS-PAGE and Western blot, phosphorylase and creatine kinase was substantially reduced in the sarcoplasmic fraction and thereafter translocated to the myofibrillar protein fraction (Fig. 2 & 3, Study I). Although the picture of Western blot showed a weak band of phosphorylase in the myofibrillar protein fraction after incubation at 30 °C (and even at lower temperatures), this difference was not large enough to be evidenced by the quantification. These results are consistent with earlier studies regarding to sarcoplasmic protein denaturation in PSE meat as summarized in Table 2. Therefore, Study I supports the claim that sarcoplasmic protein denaturation is accompanied with the loss of water-holding capacity in PSE condition (Bendall & Wismer-Pedersen, 1962). In addition, Study I showed that the precipitation of sarcoplasmic proteins was not affected during storage after the 6 h of incubation, since the amount of phosphorylase and creatine kinase did not change over 6-72 h. Therefore, it confirms the study by Schäfer et al. (2002) that the pre-rigor combination of high temperature and low pH induces sarcoplasmic protein denaturation.

The reduced water-holding capacity can be a directly result of reduced sarcomere volume as discussed in the section 2.1. The volume of the sarcomere is determined by the lattice spacing and the sarcomere length with the simplification of sarcomere as a cylinder. Table 1 in Study I shows no significant differences between sarcomere lengths measured from different temperature groups, so the differences observed in water-holding were more speculated to be in the lattice spacing. If sarcoplasmic protein denaturation is believed to be the driving force for the reduced volume of the sarcomere, then the possible mechanism behind it could be that a sedimented layer of...
denatured sarcoplasmic proteins on the surface of myofilaments decreases the net charges on them, and thereby reduces the surface interaction between water and proteins e.g. electrostatic repulsion, which results in a shorter distance between the filaments. This hypothesis has been proposed by Bendall and Wismer-Pedersen already in the 1960’s, and the suggestion has later on been repeated by others (Boles et al. 1992; Zhu et al., 2011; Bowker and Zhuang, 2015; Joo et al., 1999; Lopez-Bote et al., 1989). The present thesis clearly shows that lattice shrinks laterally in the PSE-like condition and thereby supports that sarcoplasmic protein precipitation may shield the net charges on the filaments. However, any conclusion on the overall effect of sarcoplasmic protein denaturation should also consider the effect of sarcoplasmic protein denaturation occurring in the space outside of myofibrils, such as in the drip channels as discussed later. Therefore the results in the present thesis do not agree with studies claiming that the sarcoplasmic protein denaturation is the driving force for the loss of water-holding.

**Myofibrillar protein denaturation**

In Study II, protein denaturation was found by measuring surface hydrophobicity from the myofibrils and determination of Ca$^{2+}$-ATPase activity at the myosin molecule level. After pre-rigor incubation, part of the sarcoplasmic proteins precipitated and was found subsequently in the myofibrillar fraction. Surface hydrophobicity of myofibrils was significantly higher following incubation at 40°C (Fig. 1, Study II). According to Tornberg (2005), the hydrophobic side chains in a protein molecule will tend to expose to the surface during heating. Therefore, surface hydrophobicity is a suitable indicator of protein denaturation. The increase in the surface hydrophobicity of myofibrils after incubation at 40°C can be interpreted as an indicator of protein denaturation, which will result in an altered surface interaction between myofibrils and the water molecules around. Puolanne and Halonen (2010) calculated the water-accessible surface areas (WASA) of myosin and
actin filaments to be extremely large i.e. approximately 102 000 m² (later calculations: 122 000 m²; Puolianne and Halonen, personal communication) in 1 kg of muscle. Therefore, any structural changes that interfere with the interaction between amino acids side chains and water molecules might have a profound overall effect on water-holding capacity.

However, similarly to the reduced solubility/extractability of myofibrils observed in PSE (Table 2), it is not easy to differentiate if the change in surface hydrophobicity of myofibrils is a consequence of microstructural alterations or the sediment of precipitated sarcoplasmic proteins, or both factors at the same time.

Myosin Ca²⁺ATPase activity was lost in the myofibrils after incubation at 40 °C (Fig. 2, Study II). In agreement with previous studies as listed in Table 2, the result indicated that microstructural changes occurred in the catalytic side on the myosin S1 unit. In addition to ATPase activity, a conventional way to measure the myosin denaturation, Study II was able to show structural alterations specifically on the S1/S2 junction in the PSE-like meat (Fig. 3, Study II). The chymotryptic digestion pattern has previously been shown to be able to reveal information on the structural changes in the S1/S2 regions of myosin (Takahashi, Takahashi & Konno, 2005; Yuan, Wang, Chen, Qu & Konno, 2011). Therefore, Study II highlights that temperature induced protein denaturation occurs in the region of the myosin molecule involved in formation of the cross-bridge structure of the rigor bond. Unlike the surface hydrophobicity, the precipitated sarcoplasmic proteins were unlikely to have an impact here since phosphorylase and creatine kinase completely disappeared after chymotrypsin digestion as shown by Western blot (Fig. 4, Study II). However, it cannot be excluded that some small fragments were associated to myosin. Mild heating of myosin at 35 °C for 3 h can increase the tryptic digestion susceptibility of myofibrils (Setton, Dan-Goor, & Muhlrad, 1988). In Study II, it is likely that the S1 units from PSE-like meat were completely digested into small fragments and left with the final pellet either
due to temperature induced protein aggregation or structural alterations in the catalytic site of myosin ATPase. The elevated temperature induces protein structural changes, which expose the hidden hydrophobic areas on the myosin S1 unit, and therefore the denatured S1 unit becomes more susceptible to chymotrypsin cleavage.

In the literature review section 3.1.3, the influence of myosin denaturation on the lattice spacing was discussed. Enlarged extracellular space was observed in PSE meat by light microscopy (Barbut et al., 2005), which can be a result of the additional shrinkage of lattice spacing between the filaments as proven by low angle X-ray diffraction (Irving et al., 1989, Swatland et al., 1989). Results from Study II were in line with many other studies (Table 2). If the results are interpreted in agreement with hypothesis from Offer and Knight (1998), that myofibrillar protein denaturation is the driving force for the reduced water-holding capacity in PSE meat, the denaturation possibly induces a shrinkage of lattice spacing in Study II. Structural alterations were proven to have occurred on the head region of myosin as indicated by the reduced ATPase activity and the chymotryptic digestion pattern. These results were due to the protein denaturation under PSE-like condition, which may in turn have an influence on the lattice spacing between filaments. In addition, the change in the microstructure of myosin head can also have direct influence on the interaction between amino acid side chains and water molecules. In 1 kg of muscle, S1 units in native form have 66,000 m² of water-accessible surface area. Compared to 122,000 m² contributed by all the filamental structure, the effect of S1 units in water-holding should not be underestimated (Puolanne & Halonen, 2010). Therefore, the changes in the structure of each S1 unit observed in PSE-like meat could have an overall significant impact on the water-holding.

Study I and II showed supportive results that protein denaturation in PSE meat is the causative factor accounting for the water-holding capacity. Additionally, information revealing the changes in the microstructure of S1
unit was also given. Nevertheless, it is still not possible from these studies to answer the question, whether the sarcoplasmic or the myofibrillar protein denaturation, is the driving force for the loss of water-holding. As stated in the literature review part, the simultaneous occurrence of sarcoplasmic and myofibrillar protein denaturation naturally makes it difficult to conclude on the causative relationship between either one with the loss of water-holding. This was the initial motivation for Study III, where the effect from sarcoplasmic and myofibrillar protein denaturation on water-holding were compared separately.

### 6.2. The interaction of structural and soluble proteins

*Denatured sarcoplasm increases the water-holding*

It should be noted that Study III used a model system that differs from intact meat. The ratio of sarcoplasmic to myofibrillar proteins differed. We had relatively more sarcoplasmic proteins than myofibrillar proteins, i.e. 1:1.3 compared to 1:2 in muscle (Scope, 1970). Therefore in Study III, the effect of sarcoplasmic protein denaturation on water-holding at 44 °C (Fig. 2A) was probably higher than one would expect in intact meat. According to the author’s calculation, the point around 60% of sarcoplasmic proteins in Fig. 3 (Study III), would approximately correspond to the relative composition of the protein fractions in intact meat.

Significant differences were found in water-holding between three groups of myofibrils differing in their interaction with denatured sarcoplasmic proteins (Table 1, Study III). Fig. 2A showed that temperature incubated myofibrils without any denatured sarcoplasm had the poorest water-holding capacity among all the groups. This result clearly supports the hypothesis from Offer and Knight (1988) that myofibrillar protein denaturation is the driving force for the loss of water-holding in PSE-like condition. In addition, the ATPase activity was lost notably ($P < 0.001$) following incubation at temperatures higher than 38 °C (Table 1, Study III), which was consistent with Study II,
indicating that myosin head denaturation could be related to the loss of water-holding.

This result contradicts the interpretation in previous studies that denatured sarcoplasmic proteins lowers the water-holding (Boles et al. 1992; Joo et al., 1999; Zhu et al., 2011; Bowker and Zhuang, 2015). Fig. 2A (Study III) showed that denatured sarcoplasm increased the water-holding of myofibrils following high temperature incubation post rigor, which was in agreement with Monin and Laborde (1985). Moreover, the interaction between sarcoplasm and the myofibrils during denaturation made the water-holding even better compared to the other groups. Therefore, Fig. 2A (Study III) indicated that water-holding was improved more when the denatured sarcoplasmic proteins had opportunity to subsequently precipitate onto the filaments.

Precipitated sarcoplasmic proteins increase the water-holding

The drip used in Study III contained 148±13 mg/ml of proteins, which is higher than the value of around 83-146 mg/ml that Savage, Warriss and Jolley (1990) reported. In this study, lower values were reported for samples having high drip loss suggesting that in PSE meat the denatured sarcoplasmic proteins can result in lower protein concentration in drip. Sarcoplasm contains soluble proteins and other non-protein molecules such as ions. Although the ions can also have an influence on water-holding (Hamm, 1960), it was demonstrated that the presence of proteins in the sarcoplasmic proteins, either in native or denatured state, improved the water-holding markedly (Table 1, Study III).

The presence of native sarcoplasmic protein improved the water-holding of myofibrils that had denatured at 44 °C as shown by the [MF] (Green) curve in Fig. 3 (Study III). Monin & Laborde (1985) also showed a positive impact of a sarcoplasmic extract on the water-holding of myofibrils, although it was not clear whether it was the ions or proteins that were acting. Later Wilson and
van Laack (1999) showed that compared to a PSE sarcoplasmic extract, the sarcoplasmic extract from normal pork resulted in higher water-holding capacity. They suggested that the absence of part of the proteins in the sarcoplasmic extract from PSE could be the reason. Even though heating to remove sarcoplasmic proteins does not appear to alter the ionic strength, the absence of sarcoplasmic proteins may have altered the osmotic strength (Wilson & van Laack, 1999), which could contribute to the increase of water-holding observed in [MF] (Green) curve in Fig. 3 (Study III) along the increasing amount of native sarcoplasmic proteins. With PSE-like denaturation, water-holding was improved even more, indicating that denatured sarcoplasmic proteins had a more profound positive influence on the water-holding of myofibrils than when no denaturation was involved ([MF+SP] Blue vs [MF] Green, Fig. 3, Study III).

One interesting observation that should be noticed is that a difference in water-holding (~10%) between the two experimental lines also existed when only the protein-depleted sarcoplasm was included (0% of sarcoplasmic proteins, Fig. 3, Study III). Therefore, part of the difference (total ~19%) observed in water-holding with 100% of sarcoplasmic proteins (Fig. 3) as well as the difference between [MF] and [MF+SP] (~18%) at 44 °C (Fig. 2A) were likely from the effect of the non-protein components in the sarcoplasm.

One possible explanation for this contribution of the non-protein components to water-holding may be from the change in the ionic strength. For samples with lower water-holding, myofibrils were incubated in a system with 17% of purified myofibrils and the rest was buffer. According to the author’s calculation, the ionic strength was about 88 mM in this buffer. The ionic strength in beef after rigor was in the range of 0.24 to 0.30 M (Ouali, 1992). Since removing sarcoplasmic proteins does not have an effect on the ionic strength (Wilson & van Laack, 1999), one would expect the protein-depleted drip in Study III had ionic strength around 0.24 to 0.30 M, which was more than three times compared to that of the buffer. In samples with
better water-holding, myofibrils were first mixed with protein-depleted drip, so myofibrils (8.5%) was incubated with a “solution” having ionic strength around 0.18 M. In Study III heating at 44 °C could result in a gel formation since myosin gelation starts from 30 °C at pH 5.5 (Ishioroshi, Samejima & Yasui, 1979). As myofibrils become more solubilized at higher ionic strength (Wu & Smith, 1987), a gel having better water-holding is likely to be formed by the heat-denatured proteins during temperature incubation; for a review of factors influencing myofibrillar protein gel formation, see Sun & Holley (2011).

Hamm (1962) found that the non-protein components of sarcoplasm had a positive impact on the water-holding of myofibrils. Ions such as potassium, sodium and phosphates are able to swell myofibrils and solubilize myofibrillar proteins. Lactate is also present in the post-rigor sarcoplasm, but does not seem to have an effect on myofibrillar protein solubilization (Wu & Smith, 1987). As a consequence, the extra ion species introduced into the system by adding protein-depleted drip may have improved the gelation properties of the myofibrillar proteins during the denaturation process.

Precipitated sarcoplasmic proteins shrink the lattice spacing

In the review part, the importance of the lattice spacing in relation to the amount of water that can stay within myofibrils has been stressed. Considering sarcoplasmic protein denaturation vs. myofibrillar protein denaturation, no matter which one is actually the causative reason for the reduced water-holding, the denaturation is believed to first shrink the sarcomere laterally, and thereafter the water will be expelled to the extracellular space from where it is easily lost as drip.

By X-ray diffraction, it was shown that the presence of denatured sarcoplasmic proteins in skinned muscle fibres compressed the lattice spacing significantly (Table 1 & Fig. 4B, Study III).
After the skinning procedure to remove the original sarcoplasm from the fibres before temperature incubation, sarcoplasm and protein depleted-sarcoplasm were brought back to the fibre bundles and they were subjected to 21 and 44 °C of incubation. Therefore, the independent influences of sarcoplasmic and myofibrillar protein denaturation on lattice spacing were compared.

The black bars in Fig. 4B (Study III) showed that myofibrillar protein denaturation after post-rigor temperature incubation will shrink the lattice spacing. Together with Study II, it supports the hypothesis that myosin denaturation in PSE-like condition will alter the microstructure of myosin head regions, which results in a shrinkage of the lattice spacing. It is assumed that the water-holding capacity of myofibrils (Fig. 2A) reflects the water-holding of fibres bundles. Sarcomere lengths were fixed as the meat samples used were in rigor mortis. By an approximate calculation, the reduction in the lattice spacing accounted for 15% of reduction in the volume of the denatured myofibril compared to native myofibrils. It is thus in parallel with the difference in found in the water-holding of myofibrils, which was 16% of reduction in the denatured myofibrils compared to the native myofibrils (in [MF] curve: 44 vs 21°C; Fig. 2A, Study III). Therefore, the present study provided for the first time X-ray diffraction result, which confirms the hypothesis by Offer and Knight (1988) that in PSE meat the myosin denaturation is the causative factor for the loss of water-holding due to the substantial shrinkage in the lattice spacing.

There was an additional shrinkage in the lattice spacing due to the precipitation of sarcoplasmic proteins as illustrated in Fig. 4B (Study III). It shows that precipitated sarcoplasmic proteins will compress the lattice laterally, which could have been caused by shielding of net charges.

Part of the sarcoplasmic proteins started to lose their solubility when the PSE-like condition was introduced (Study I) which has been also observed in other
studies as well as listed in Table 2. The pI values of the most abundant sarcoplasmic proteins are typically higher than 5.6. Therefore, when coagulated and precipitated onto the surface of the myofilaments, they are speculated to add positive charges and thus result in a decrease of the overall negative net charges. As a consequence, the reduced net charges could reduce the electrostatic repulsion between the filaments and thereby compress the filaments as shown by the X-ray data.

Assuming that the reduced lattice spacing will always result in the decrease in the overall water-holding, the additional shrinkage in lattice spacing due to precipitation of sarcoplasmic protein appears to contradict the results in Fig. 2A (Study III) because the water-holding was actually improved by the precipitated sarcoplasmic proteins. From the literature it is difficult to find any relevant explanation for this. Therefore, a new hypothesis was proposed in the current thesis.

6.3. New hypothesis explaining the role of sarcoplasmic protein denaturation on water-holding

The current author proposes that in PSE-like conditions, the sarcoplasmic proteins denature and aggregate in the intramyofibrillar, intermyofibrillar and extracellular space. The overall combined effect of the denatured sarcoplasmic proteins is to improve the water-holding capacity by a process that is compartmentalized as illustrated by Fig. 5 (Study III).

As summarized by Table 1 in the review part, apart from the bound water, there are three distinct compartments where water can stay, which are 1) intramyofibrillar space, 2) intermyofibrillar space and 3) extracellular space as also illustrated in Fig. 5 (Study III).

At Level 1), denatured sarcoplasmic proteins coagulate on the surface of filaments, and thereby reduce the lattice spacing, while at Level 2) and 3) the denatured sarcoplasmic proteins will build up a gel-like network, which traps water that has been expelled from Level 1). The positive effects from 2) and 3)
on the water-holding counteract with the negative effects on water-holding imposed by the denaturation of myofibrils and the sarcoplasmic protein precipitation at Level 1).

In the new hypothesis not only the effect from the denatured sarcoplasmic proteins inside of myofibrils is discussed, but also other spaces are included such as: the space between myofibrils (intermyofibrillar space), and the space between the fibres. The reason to take these three compartments into consideration is because of the drip channel formation in the PSE condition. In the review, the time frame of drip channel formation during conversion of muscle to meat was described in section 1.4.

Sarcoplasm originates from the intermyofibrillar space and then migrates hierarchically as from intermyofibrillar $\rightarrow$ intramyofibrillar $\rightarrow$ extracellular space, where it is the least strongly held and susceptible to be lost afterwards. The extracellular space gradually develops post mortem and the gaps between fibres can be seen at 24 h post mortem by microscopy (Offer & Cousin, 1992). In meat with poor water-holding such as PSE, the onset of drip channel formation can be even earlier and the interfibre space will be larger compared to normal meat (Barbut et al., 2005; Irving et al., 1989; Swatland et al., 1989). Therefore, proteins in the sarcoplasm that has migrated to the intramyofibrillar and extracellular space will also denature and aggregate simultaneously with the sarcoplasmic proteins within the intermyofibrillar space.

Sarcoplasmic proteins are globular proteins that will unfold upon increasing temperature and subsequently form a gel as discussed in the review part, section 3.2.1. The gel could be porous and good at trapping the water according to the microscopy shown by Miyaguchi, Hayashi, and Sakamoto (2007). Therefore, when observing the effect of the denatured soluble proteins on the water-holding capacity, the shrinkage of lattice spacing does not necessarily correlate with the loss of water-holding. The influences of
denatured sarcoplasmic proteins within the other compartments on the water-holding should also be considered.

7. Conclusions

Pre-rigor incubation at 40 °C for 6 h and post-rigor incubation at temperatures higher than 38 °C at pH 5.6 reduces the water-holding capacity of muscle and myofibrils. In addition, protein denaturation in the sarcoplasmic fraction is indicated by:

- The reduced solubility
- The precipitation of glycogen phosphorylase and creatine kinase

In addition protein denaturation in the myofibrils is indicated by:

- The reduced Ca\(^{2+}\) ATPase activity
- The increased surface hydrophobicity
- The structural alterations on S1/S2 region by chymotryptic digestion pattern

The current thesis confirms that in PSE-like condition, the denaturation of myofibrils is the driving force for the loss of water-holding capacity by a mechanism that involves reducing the lattice spacing. It is shown that the sarcoplasmic protein precipitation adds additional shrinkage to the lattice spacing but still imposes an overall positive impact on the water-holding.

In the thesis a new hypothesis is proposed:

In conditions of heat-induced protein denaturation such as occurring in PSE, these events occur in parallel. Within the intermyofibrillar space sarcoplasmic proteins precipitate and additional shrink the lattice spacing. In the intermyofibrillar and extracellular space, denatured sarcoplasmic proteins form a network, which will improve the water-holding capacity. The denaturation of sarcoplasmic protein counteracts the negative influence from the denatured myofibrils on water-holding.
8. Future perspectives

- The thesis shows that precipitated sarcoplasmic proteins shrink the lattice spacing under PSE-like condition. However, the mechanism behind it is still unknown. To test if it is an effect of “shielding of charges”, which means that the layer of precipitated sarcoplasmic proteins shields the negative net charges on the myofilaments, and thereby results in less repulsive forces and a subsequent reduction in the lattice spacing, the net charges on the myofilaments structure before and after sarcoplasmic protein precipitation can possibly be measured by Donnan potential.

- The hypothesis proposed by the current thesis stresses that the denatured sarcoplasmic proteins are affecting water-holding in a compartmentalized way. To provide more solid evidence for this:

  1) The locations of the precipitated sarcoplasmic proteins within intramyofibrilar, intermyofibrillar and extracellular space can be visualized, for instance by immunohistological techniques blotting against glycogen phosphorylase or creatine kinase.

  2) The influence of denatured sarcoplasmic proteins on the water mobility within the muscle structure can be studied by LF-NMR $^1$H $T_2$ relaxometry.

- The positive impact of sarcoplasmic proteins on the water-holding of meat should be noticed in the industry. Loosing sarcoplasmin not only will result in a reduction in the final fresh weight of meat but also a loss of water-holding capacity of myofibrils.
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