Department of Food and Nutrition
University of Helsinki
Finland

Myofibril-bound calpains: Mechanisms on degradation of myofibrillar proteins and meat tenderization

Jian Lyu

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in Biocenter 2 hall 2041, Viikki
on 21 March 2022, at 12 noon.

Helsinki 2022

Custos

Associate Professor Per Ertbjerg
Department of Food and Nutrition
University of Helsinki
Helsinki, Finland

Supervisor

Associate Professor Per Ertbjerg
Department of Food and Nutrition
University of Helsinki
Helsinki, Finland

Reviewers

Professor Steven Lonergan
Department of Animal Science
Iowa State University
Ames, United States

Associate Professor Brad Kim
Department of Animal Sciences
Purdue University
West Lafayette, United States

Opponent

Associate Professor Margrethe Therkildsen
Department of Food Science
University of Aarhus
Aarhus, Denmark

ISBN 978-951-51-8005-6 (paperback)

ISBN 978-951-51-8006-3 (PDF)

ISSN 0355-1180

Unigrafia

Helsinki 2022

Table of Contents

Abstract ... i

Acknowledgements ... iii

List of original publications ... v

Abbreviations ... vi

1. Introduction ... 1

2. Literature review ... 4

2.1. Calpain and its translocation during postmortem storage ... 4

2.1.1. Characteristics and localization of calpain system ... 4

2.1.2. Ca^{2+} and activation of calpains ... 6

2.1.3. Evidence of myofibril-bound calpains ... 7

2.2. Early postmortem changes and proteolytic tenderization in meat ... 9

2.2.1. Early postmortem changes in meat ... 9

2.2.2. Proteolytic tenderization of meat during postmortem storage ... 10

2.2.2.1. Calpain mediated meat tenderization ... 10

2.2.2.2. Other tenderness-related enzymes ... 13

2.2.3. Role of postmortem proteolysis on water-holding ... 15

2.3. Calpain activity in meat and its influencing factors ... 17

2.3.1. Measurements for activity of calpains during storage ... 17

2.3.2. Content and activity of myofibril-bound calpains ... 20

2.3.3. Factors influencing calpain activity ... 22

2.4. Hypothesis for meat tenderization caused by myofibril-bound calpains ... 24

2.4.1. Current explanation ... 24

2.4.2. Mechanisms of myofibril-bound calpains on meat tenderization ... 25

3. Objectives ... 27

4. Materials and methods ... 28

4.1. Sample processing ... 28

4.2. Physical analysis ... 29

4.3. Biochemical analysis ... 31

4.4. Statistical analysis ... 33

5. Summary of results 34
5.1. Changes in free Ca^{2+} concentration during storage and its role in the binding of calpain-2 to myofibrils 34
5.2. Translocation of calpains and changes of activity of myofibril-bound calpains during storage 35
5.3. Effect of pre-rigor temperature on meat quality regarding proteolysis and WHC of meat 38
6. General discussion 39
6.1. Ca^{2+} and its role in the formation of myofibril-bound calpains 39
6.2. Hypothesis for postmortem proteolysis by myofibril-bound calpains 43
6.3. Effects of myofibril-bound calpains induced proteolysis and protein denaturation on meat quality 47
7. Conclusions 51
8. Future perspectives 52
References 53

Abstract

The aim of the thesis was to investigate the effects and mechanisms of myofibril-bound calpain-1 and calpain-2 on the degradation of myofibrillar proteins and meat tenderization during postmortem storage.

The role of Ca^{2+} in the process of purified calpain-2 becoming bound to the myofibrils isolated from 12-h porcine *longissimus thoracis* (LT) muscles and the proteolytic activity of myofibril-bound calpains to degrade desmin were investigated. There were calpains bound to isolated myofibrils and the myofibril-bound calpains were proteolytically active when sufficient Ca^{2+} was added. Purified calpain-2 can become relatively tightly bound to myofibrils, a process which was induced by Ca^{2+}. The binding of purified calpain-2 to isolated myofibrils occurred in a Ca^{2+} concentration-dependent manner. After the binding process, the myofibril-bound calpain-2 was proteolytically active and thus able to degrade desmin.

In the porcine LT muscles stored at 2 °C for 1, 3, 6, 9 and 12 days, the content of sarcoplasmic calpain-1 was observed to decrease, while the myofibril-bound calpain-1 content increased until day 6 followed by a gradual decrease with subsequent storage. These results suggest that calpain-1 gradually translocates from sarcoplasm to myofibrils during early postmortem storage. Desmin in isolated myofibrils was degraded by myofibril-bound calpains upon incubation with Ca^{2+} in the order of 5 mM > 0.05 mM > 0. Ca^{2+} titration curves indicated that myofibrils contain two distinct proteolytic activities corresponding to the known Ca^{2+} requirements for calpain-1 and calpain-2, respectively. The results suggest that both calpain-1 and calpain-2 will be bound to myofibrils during storage and subsequently degrade desmin.

The effects of pre-rigor temperature on the calpain-1 distribution between sarcoplasm and myofibrils and activity of calpains were investigated by incubating pre-rigor LT muscles at 14, 22, 30 and 38 °C for 6 h followed by 2 h incubation at 14 °C and then storage for 1 and 4 days at 2 °C. The free Ca^{2+} concentration in the sarcoplasm, the translocation of clapain-1 from the sarcoplasm to the myofibrils and degradation of myofibrillar proteins increased significantly early postmortem with increased pre-rigor temperature. However, calpain-1 lost its proteolytic activity in muscle at the highest temperature (38 °C). Decreased water-holding capacity (WHC) of myofibrils and increased purge loss from meat pieces were observed with the increase of temperature. Furthermore, WHC of myofibrils, degradation

of desmin and troponin T, and myofibril fragmentation increased after incubation of myofibrils with Ca^{2+}, indicating that proteolysis caused by myofibril-bound calpains has the potential to improve WHC of myofibrils.

In conclusion, the results of the current thesis suggest that Ca^{2+} induces both calpain-1 and calpain-2 to bind to myofibrils in a concentration-dependent manner. Calpains gradually translocate from the sarcoplasm to myofibrils during storage and the translocation will be accelerated by an increased pre-rigor temperature. The myofibril-bound calpains are proteolytically active to degrade myofibrillar proteins in the presence of Ca^{2+} and thereby leading to meat tenderization.

Acknowledgements

The work in the current thesis was carried out in Meat Technology group, Department of Food and Nutrition, Faculty of Agriculture and Forestry, University of Helsinki from October, 2017 to November, 2021. Financial resource comes from the scholarship provided by the China Scholarship Council. Chancellor's Travel Grant / Doctoral Programme in Food Chain and Health supported me to attend international conferences. These financial supports are greatly appreciated and acknowledged.

My deepest gratitude goes to my supervisor, Associate Professor Per Ertbjerg for all the guidance and support from the application of PhD student position to the completion of my doctoral study. Thank you for always being nice and patient to the problems occurring in my study. You are so intelligent and can always give useful suggestions and help me solve the troubles. I was inspired a lot during our academic discussion. You are very scientific rigorous and always know how to make the study more logic and complete. I am very lucky to have the chance to learn these treasurable characters from you.

I would like to give my sincere gratitude to my teacher and friend, Professor Emeritus Eero Puolanne. You are very considerate and do your best to help me in my study and life. You are always being encouraging and supportive and give me more courage and strength to face difficulties. I have always been enlightened and guided by your wisdom during our conversation.

I owe my acknowledgement to Professor Steven Lonergan and Associate Professor Brad Kim for their valuable comments and suggestions to the pre-examination of the current thesis. I kindly thank University Lecturer Tuula Sontag-Strohm and Kirsi Jouppila for being as the follow-up members of my doctoral study.

I am truly grateful to my nice colleagues in our group Yulong Bao, Yuemei Zhang, Binbin Li, Xueshen Zhu, Luigi Pomponio, Yuduan Diao, Arianna Magro and Ingrid Roner, thank you for your assistance and creating a pleasant atmosphere in our office and lab. I owe my warm gratitude to my dear friends in our department: Yaqin Wang, Xin Huang, Fengyuan Liu, Hongbo Zhao, Ruoxi Zhang, Xiaocui Han, Zhen Yang, Chang Liu, Chong Xie and Yan Xu, etc, thank you for your help and the memorable time we spent together. I kindly thank technical support from Mikko Kangas, Taru Rautavesi, Outi Brinck, Miikka Olin, Jutta Varis, and other technicians. You are so nice and patient to help me with all the troubles in lab. I am also grateful to all colleagues I worked with in the lab.

I also want to thank my precious friends Ran Li, Man Hu, Yufan Yin, Changyi Lu, Enpei Zhang, Yinyin Wang, Zhipeng Tang, Ming Guo and Zhenyang Wei etc. It is a great experience to share my life in Finland with you. Your friendships make it more colorful and meaningful.

Finally, my warmest thanks go to my family (in Chinese). 感谢爸妈一直以来的培养和呵护，你们无条件的支持让我能无所畏惧，充分自由的、专注的发展自我。谢谢妹妹的理解和鼓舞，很庆幸一直以来有你分享彼此成长路上的点滴。感谢所有的家人们，你们的爱陪伴我勇敢坚定地走过那些或开心，或平静，或焦虑的时刻，一直给予我最深厚、最安全的支撑。

吕健 Jian Lyu

Helsinki, January

Jian Lyu

List of original publications

Paper I

Lyu, J., & Ertbjerg, P. (2021). Ca^{2+}-induced binding of calpain-2 to myofibrils: Preliminary results in pork *longissimus thoracis* muscle supporting a role on myofibrillar protein degradation. Meat Science, 172, 108364.

Paper II

Lyu, J., & Ertbjerg, P. (2022). Sarcoplasmic and myofibril-bound calpains during storage of pork *longissimus* muscle: New insights on protein degradation. Food Chemistry, 131347.

Paper III

Lyu, J., Puolanne, E. & Ertbjerg, P. (Manuscript). Effect of pre-rigor temperature incubation on myofibril-bound calpains activity, protein degradation and meat properties in pork *longissimus* muscle.

List of authorships

I: The study was planned by Jian Lyu and Per Ertbjerg together. The experimental analyses and manuscript preparation were done by Jian Lyu. Per Ertbjerg gave comments and suggestions during the manuscript preparation.

II: The study was planned by Jian Lyu and Per Ertbjerg together. The experimental analyses and manuscript preparation were done by Jian Lyu. Per Ertbjerg gave comments and suggestions during the manuscript preparation.

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

Abbreviations

ATP	Adenosine triphosphate
BODIPY-FL	4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-propionic acid
BSA	Bovine serum albumin
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene-bis(oxyethylenenitrilo)tetraacetic acid
FITC	Fluorescein isothiocyanate
LD	*M. longissimus dorsi*
LT	*M. longissimus thoracis*
LTL	*M. longissimus thoracis et lumborum*
MES	2-(N-Morpholino) ethanesulfonic acid hydrate
NS	Not significant
PM	*M. pectoralis major*
PSE	Pale, soft and exudative
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SM	*M. semimembranosus*
Std	Standard deviation
TCA	Trichloroacetic acid
Tris	Tris(Hydroxymethyl)aminomethane
WB	Western blot
WHC	Water-holding capacity

1. Introduction

Meat tenderness is one of the most important eating quality attributes (Shackelford, Wheeler, Meade, Reagan, Byrnes, & Koohmaraie, 2001). Degradation of key structural proteins during postmortem storage plays an important role in tenderization of meat (Huff-Lonergan & Lonergan, 2005; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). The contribution of the calpain system in postmortem degradation of cytoskeletal proteins and in meat tenderization have been generally accepted (Bhat, Morton, Mason, & Bekhit, 2018; Koohmaraie & Geesink, 2006). The most studied are calpain-1 and calpain-2, and their specific inhibitor calpastatin. The activity of calpain-1 and calpain-2 during aging is regulated by the concentration of free Ca^{2+} in the sarcoplasm, calpastatin, autolysis, phosphorylation and its intracellular localization (Perrin & Huttenlocher, 2002).

It has been reported that the extractable activity for calpain-1 as measured in vitro (measured following partly purification) decreased rapidly in beef during early postmortem storage (Camou, Marchello, Thompson, Mares, & Goll, 2007; Koohmaraie, Seidemann, Schollmeyer, Dutson, & Crouse, 1987). In addition, autolysis of calpain-1 was found to occur in pork in less than one day (Pomponio & Ertbjerg, 2012; Zhang & Ertbjerg, 2018). The decrease of extractable activity as assayed in vitro and the emergence of autolyzed forms of calpains are recognized as be the indicators for proteolytic activity of calpains and their involvement in meat tenderization during postmortem storage (Goll, Thompson, Li, Wei, & Cong, 2003; Melody, Lonergan, Rowe, Huiatt, Mayes, & Huff-Lonergan, 2004). However, only little proteolysis of myofibrillar proteins was found during the 24 to 48 h postmortem storage as observed by very limited degradation of desmin (Kristensen & Purslow, 2001), troponin T (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson, & Robson, 1996) and titin (Fritz & Greaser, 1991). In addition, the extractable activity of calpain-1 reduced more rapidly compared with that of calpastatin (Boehm, Kendall, Thompson, & Goll, 1998), suggesting that even though the level of free Ca^{2+} in postmortem muscle is high enough to activate calpain-1, its proteolytic activity could still be inhibited by calpastatin to a large extent. Taken together, it is difficult to explain the time difference between the substantial decrease in extractable activity of calpain-1 and degradation of myofibrillar proteins without consideration of other proteases. More detailed information on the process for proteolytic degradation of myofibrillar proteins induced by calpains and the resultant contribution to tenderness of meat is therefore needed.

Calpains are located exclusively intracellularly and are mainly free in sarcoplasm (Goll et al., 2003), although association of part of the calpains with subcellular organelles like myofibrils (Ishiura, Sugita, Nonaka, & Imahori, 1980), plasma membranes (Zhang et al., 2021a; Hood, Logan, Sinai, Brooks, & Roszman, 2003) and mitochondria (Thompson, Hu, Lesnefsky, & Chen, 2016) was observed in some studies. Translocation of calpain-1 from the sarcoplasm to myofibrils during storage has been observed in a few studies (Boehm et al., 1998; Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001b; Melody et al., 2004; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004b). In addition, it has been reported by Zeng, Li, & Ertbjerg (2017) that a higher degree of desmin degradation caused by myofibril-bound proteases with increased Ca^{2+} concentrations was observed and the myofibril-bound proteases were suggested to be calpain-1 and calpain-2. The observations of the increased content of myofibril-bound calpain-1 in postmortem meat (Boehm et al., 1998; Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001a; Delgado et al., 2001b; Melody et al., 2004; Rowe et al., 2004b) and the calpain-like proteolytic activity activated by Ca^{2+} within myofibrils (Zeng et al., 2017) may provide new insights on how calpains work during postmortem storage. However, there is very limited information about the characteristics of myofibril-bound calpains.

It is generally recognized that calpain-1 plays a role in proteolysis of myofibrillar proteins and the resultant tenderization during postmortem storage of meat (Huff Lonergan, Zhang, & Lonergan, 2010; Koohmaraie & Geesink, 2006), whereas the contribution of calpain-2 is still under debate due to its high Ca^{2+} requirement which is 400-800 μM for half-maximal activity (Goll et al., 2003). However, the extractable activity of calpain-2 has been reported to reduce to some extent during meat aging (Boehm et al., 1998; Camou et al., 2007; Phelps et al., 2016). Also, partly autolyzed calpain-2 has been observed in pork during refrigerated storage (Pomponio, Lametsch, Karlsson, Costa, Grossi, & Ertbjerg, 2008). The activation of calpain-2 can be increased by higher temperature of muscle during early postmortem storage (Liu, Ruusunen, Puolanne, & Ertbjerg, 2014; Pomponio & Ertbjerg, 2012), by the freezing-thawing process of meat (Colle et al., 2018; Zhang & Ertbjerg, 2018) and by extended aging time (Colle & Doumit, 2017). Hence, it would be reasonable to investigate the proteolytic activity of calpain-2 during storage and its role in meat tenderization.

Proteolytic potential of calpains is influenced by the temperature and pH of muscle (Hwang, Park, Cho, & Lee, 2004; Mohrhauser, Lonergan, Huff-Lonergan, Underwood, & Weaver, 2014). In pork, PSE (pale, soft, exudative) muscle is more likely to be found due to the high pre-rigor temperature with fast

pH decline induced by rapid glycolysis (Bendall & Wismer-Pedersen, 1962). Some studies have reported that a high pre-rigor temperature leads to more tender meat at an early storage due to the earlier activation of calpains (Liu et al., 2014; Dransfeld, 1994). However, proteins and enzymes in meat tend to denature under this condition (Kim, Stuart, Nygaard, & Rosenvold, 2012; Kim et al., 2010). Thus, the role of calpains in tenderization of meat that were exposed to a high pre-rigor temperature is still not well understood.

In the current thesis, pre-rigor incubation with increased temperature (14, 22, 30 and 38°C) and refrigerated storage were applied to study the translocation of calpain-1 and calpain-2 from the sarcoplasm to the myofibrils, and the proteolytic activity of myofibril-bound calpains on degradation of myofibrillar proteins. Detailed knowledge on the binding of calpains to myofibrils was provided. The aim was to provide new insights on the contribution of myofibril-bound calpains to the meat tenderization.

The literature review in the current thesis is first presented in Chapter 2 as: 1) characteristics of the calpain system and Ca^{2+} induced translocation of calpains; 2) early postmortem changes in muscle and meat tenderization caused by calpains and other endogenous enzymes; 3) activity of sarcoplasmic and myofibril-bound calpains and their influencing factors; 4) hypothesis for meat tenderization. The objectives of current thesis are listed in Chapter 3. Materials and methods are briefly described in Chapter 4. A summary of results is shown in Chapter 5 and a general discussion is presented in Chapter 6. Finally, conclusions and future perspectives are given in Chapter 7 and 8, respectively.

2. Literature review

2.1. Calpain and its translocation during postmortem storage

2.1.1. Characteristics and localization of calpain system

The calpain system is a large family of intracellular Ca^{2+}-dependent cysteine proteases (Sorimachi, Saido, & Suzuki, 1994). The most studied in the calpain system are calpain-1 and calpain-2 (also known as μ- and m-calpain) and their specific inhibitor calpastatin. They are believed to play an important role in proteolysis of myofibrillar proteins and thereby meat tenderization during storage (Huff Lonergan et al., 2010; Kemp & Parr, 2012). In addition, some activity of calpain-3 (p94) (Ilian, Bekhit, & Bickerstaffe, 2004a; Ilian, Bekhit, Stevenson, Morton, Isherwood, & Bickerstaffe, 2004; Yang, Chen, Jia, & Zhao, 2012) and calpain-10 (Ilian, Bekhit, & Bickerstaffe, 2004b) have been reported in some studies but their contribution to meat tenderization is still controversial.

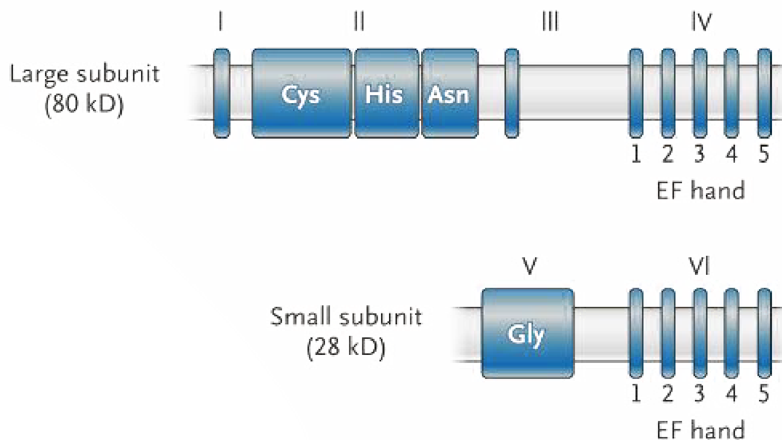

Fig. 1. Schematic representation demonstrating domain structure of calpain subunits. The large subunit (80 kDa) and small subunit (28 kDa) contain four and two domains, respectively. Six and five EF hands are shown in the large and small subunits, respectively (Zatz & Starling, 2005).

Calpain-1 and calpain-2 are two very similar isozymes. They are heterodimers composed of an identical 28 kilodalton (kDa) regulatory subunit and a 80 kDa catalytic subunit that shares 55-65% sequence homology (Fig. 1). The large and small subunits comprise four (I – IV) and two (V – VI) domains, respectively (Blanchard et al., 1997). Domain II is the catalytic site and has two conserved sites to bind Ca^{2+}. Domain IV and VI are Ca^{2+}-binding domains and each contains five EF-hand motifs

(Khorchid & Ikura, 2002; Sorimachi & Suzuki, 2001). The interaction between the two fifth EF-hand is responsible for the heterodimerization of the large and small subunits in calpains (Moldoveanu, Hosfield, Lim, Elce, Jia, & Davies, 2002). Calpains can be divided into two categories, based on their distribution: ubiquitous (such as calpain-1, calpain-2 and calpain-10) and tissue specific (such as calpain-3) calpains (Horikawa et al., 2000; Sorimachi et al., 1994). Immunolocalization has been used intensively as a technique for studying localization of calpain system in muscle (Ishiura et al., 1980; Melody et al., 2004). Calpains are located exclusively intracellularly as measured by immunolocalization (Goll et al., 2003). They are predominantly free in sarcoplasm and more precisely near the Z-disk and I-band and in very small amount near the A-band (Goll et al., 2003; Vosler, Brennan, & Chen, 2008). However, part of the calpains associate with subcellular organelles such as myofibrils (Delgado et al., 2001b; Ishiura et al., 1980), cell membranes (Hood et al., 2003; Mellgren, 1987), mitochondria (Kar, Samanta, Shaikh, Chowdhury, Chakraborti, & Chakraborti, 2010; Ozaki, Tomita, Tamai, & Ishiguro, 2007; Thompson et al., 2016), endoplasmic reticulum and Golgi apparatus (Hood, Brooks, & Roszman, 2004; Hood et al., 2003).

Calpain-1 and calpain-2 are specifically inhibited by endogenous calpastatin (110 kDa) which has four repeating inhibitory domains and each of the four domains contains three subdomains, named A, B and C. Every inhibitory domain can bind one calpain molecule and thereby each calpastatin molecule has the potential to inhibit four calpain molecules (Hanna, Garcia-Diaz, & Davies, 2007; Maki, Takano, Mori, Sato, Murachi, & Hatanaka, 1987). The interaction between calpains and calpastatin is Ca^{2+}-dependent, and its Ca^{2+} requirement is lower than that required for proteolytic activity of intact calpain-1 and calpain-2, and autolyzed calpain-2. The Ca^{2+} requirement for activity of autolyzed calpain-1 is close to that of interaction between calpastatin and calpain-1 (Kapprell & Goll, 1989). However, calpastatin can also be degraded by calpains and the degradation products of calpastatin could still show some inhibitory activity towards calpains (Doumit & Koohmaraie, 1999; Mellgren, Mericle, & Lane, 1986). Calpastatin can regulate the rate and extent of postmortem proteolysis and tenderization by its inhibition on calpain (Geesink & Koohmaraie, 1999b). It has been reported that calpastatin activity was positively correlated with shear force (Warner Bratzler) in aged meat (de Moura Souza, Coutinho, Ramos, de Oliveira, Lonergan, & Delgado, 2019; Wulf, O'Connor, Tatum, & Smith, 1997) and calpastatin activity at 24 h has been used as an indicator in some studies to predict meat tenderness (Whipple, Koohmaraie, Dikeman, Crouse, Hunt, & Klemm, 1990.

2.1.2. Ca^{2+} and activation of calpains

Ca^{2+} plays an important role in the regulation of calpains. Ca^{2+} is a trigger for proteolytic activity of calpains due to that binding of Ca^{2+} to calpains lead to conformational changes (Moldoveanu et al., 2002). Except the EF-hands in domains IV and VI, two additional Ca^{2+} binding sites were identified in the peptides loops of domains IIa and IIb. Both domains IV and VI bind four Ca^{2+}, and domains IIa and IIb each bind one Ca^{2+} (Hanna, Campbell, & Davies, 2008). It has been shown by the crystal structures of Ca^{2+}-free calpain-2, that the catalytic residues in domain I and II are not correctly aligned, which is probably due to the constraints imposed by the circular arrangement of domains (Moldoveanu et al., 2002). The association of Ca^{2+} to calpains was thought to release the constraints and realignment of the active site cleft and consequently lead to activation of calpains (Khorchid & Ikura, 2002; Moldoveanu et al., 2002; Nagaraj, Anilakumar, & Santhanam, 2002).

Calpain-1 and calpain-2 have similar proteolytic activity on myofibrillar proteins but differ in their Ca^{2+} requirements. The reported Ca^{2+} requirements on half-maximal activity of calpain-1 and -2 are 3-50 and 400-800 μM, respectively, which are much higher than the 50-300 nM of physiological Ca^{2+} concentration in the sarcoplasm (Goll et al., 2003). However, it has been reported that the Ca^{2+} concentration can increase to 50-400 μM in the sarcoplasm of meat during postmortem storage (Colle et al., 2018; Hwang et al., 2004; Ji & Takahashi, 2006; Zhang & Ertbjerg, 2018) which is sufficient for the activation of capain-1 and partial activation of calpain-2. Parrish, Selvig, Culler, & Zeece (1981) used atomic absorption spectrophotometry and found that there is 630-970 μM of free Ca^{2+} in bovine *longissimus* muscles after storage, which is sufficient for full activity of calpain-2. Sarcoplasmic reticulum and mitochondria have been assumed to play an important role in regulation of intracellular Ca^{2+} concentration in living cells (Whiting, 1980). Decreased pH and high muscle temperature after slaughter cause a lower stability of sarcoplasmic reticulum and mitochondria and then Ca^{2+} leaks into the sarcoplasm, while the Ca^{2+}–ATPase pump could not transport the Ca^{2+} back into sarcoplasmic reticulum, which ultimately leads to an increased sarcoplasmic Ca^{2+} level during postmortem storage (Cornforth, Pearson, & Merkel, 1980; Ji & Takahashi, 2006; Whiting, 1980).

Calpains can be autolyzed in the presence of Ca^{2+}. The 80 kDa subunits of calpain-1 and calpain-2 reduce their mass to 78 kDa, while calpain-1 will be reduced further to 76 kDa during the autolysis. Autolysis can be seen as an indicator that calpains are (or have been) proteolytically active, however, the absence of autolysis does not guarantee that calpains have not been active (Cong, Goll, Peterson, & Kapprell, 1989). It has also been reported that autolysis will reduce the Ca^{2+} requirements for

proteolytic activity of calpains (Edmunds, Nagainis, Sathe, Thompson, & Goll, 1991; Elce, Hegadorn, & Arthur, 1997).

2.1.3. Evidence of myofibril-bound calpains

Except for the Ca^{2+} concentration and the inhibitor calpastatin, differential intracellular localization of calpains was also reported to influence the regulation of calpains (Zhang, Wang, & Peng, 2021b; Gil-Parrado et al., 2003). Calpains are widely distributed within the cytoplasm in unstimulated cells (Garcia, Bondada, & Geddes, 2005). However, redistribution of calpains was found in cells in response to stimulation by signals such as a rise in sarcoplasmic Ca^{2+} (Baek, Yu, Kim, Na, & Kwon, 2016; Chang et al., 2015; Hernando, Inserte, Sartório, Parra, Poncelas-Nozal, & Garcia-Dorado, 2010; Molinari & Carafoli, 1997). The translocation of calpains from the cytosol to the plasma membrane has been well documented in cardiomyocytes (Zhang et al., 2021a; Lu, Feng, Tang, Zhou, Gao, & Ren, 2020; Rios et al., 2020; Hernando et al., 2010), erythrocytes (Michetti et al., 1996; Glaser, Schwarz-Benmeir, Barnoy, Barak, Eshhar, & Kosower, 1994) and other cells (Garcia et al., 2005), and redistribution to the mitochondria was also observed in several reports (Chelko et al., 2021; Ni et al., 2015; Badugu, Garcia, Bondada, Joshi, & Geddes, 2008; Moshal et al., 2006; Ozaki, Yamashita, & Ishiguro, 2008). The increase in Ca^{2+} concentration induced calpains to translocate to the cell membrane or mitochondria and thereafter degrade substrate proteins for some physiological purpose. Translocation of calpain-1 from cytosol to other sub-organelles decreased by treatment with EDTA or EGTA, indicating that Ca^{2+} plays an important role in the redistribution of calpains (Chelko et al., 2021; Ariyoshi et al., 1993). In addition, Hernando et al. (2010) reported that translocation of calpains in response to the Ca^{2+} increase during myocardial ischemia was not sufficient for activation of calpains. The restricted translocation of calpains was thus proposed to be a necessary step for the activation of calpains under Ca^{2+} concentration in living cells (Hernando et al., 2010). Molinari & Carafoli (1997) also reported that the association of the calpain to plasm membrane is likely to be a step of activation pathway in vivo. This translocation was also suggested to serve as a way regulating substrates available for proteolysis by the proteases following their activation (Spencer & Tidball, 1996). Similarly, the proteolysis of proteins in myofibrils by calpains probably follows the same pattern that calpains translocate to the myofibrils and thereafter degrade myofibrillar proteins. Some studies have shown that calpain-1 in beef (Boehm et al., 1998; Rowe et al., 2004b) and pork (Melody et al., 2004) gradually translocated from sarcoplasm to myofibrils during storage. Similar results were

found in the current thesis and the myofibril-bound calpains were proteolytically active and able to degrade myofibrillar proteins after being activated by Ca^{2+}. Evidence from studies of different animals and muscles showing that calpains bind to myofibrils during storage is summarized in Table 1.

Table 1. Summary of evidence from literature regarding the existence of myofibril-bound calpains

Species and muscle	Existence evidence	Reference
Pork LT	Increased desmin degradation after incubation of myofibrils with Ca^{2+}	(Study I)
Pork LT	WB of calpain-1 in myofibrils; Increased degradation of myofibrillar proteins and increased peptides release after incubation of myofibrils with Ca^{2+}	(Study II & III)
Beef LL	WB of calpain-1 in myofibrils	de Oliveira, Delgado, Steadham, Huff-Lonergan, & Lonergan (2019)
Pork LTL	Increased desmin degradation and increased peptides release after incubation myofibrils with Ca^{2+}	Zeng et al. (2017)
Beef LT	WB of calpain-1 and calpain-2 in myofibrils	Neath et al. (2007)
Beef LM	WB of calpain-1 in myofibrils	Rowe et al. (2004b)
Pork LD, PM, SM	WB of calpain-1 in myofibrils	Melody et al. (2004)
Lamb LTL	WB of calpain-1 and calpain-3 in myofibrils	Ilian et al. (2004)
Lamb LTL	WB of calpain-1 and calpain-3 in myofibrils	Ilian, Bekhit, & Bickerstaffe (2004a)
Lamb LTL	WB of calpain-1 and calpain-3 in myofibrils; Immunofluorescence microscopy of calpain-3 in myofibrils	Ilian, Bickerstaffe, & Greaser (2004)
Lamb LTL	WB of calpain-10 in myofibrils	Ilian, Bekhit, & Bickerstaffe (2004b)
Lamb LD and *infraspinatus*	WB of calpain-1 in myofibrils	Delgado et al. (2001a)
Lamb LD	WB of calpain-1 in myofibrils; Increased ^{14}C release after incubation of myofibrils with ^{14}C labeled casein and Ca^{2+}	Delgado et al. (2001b)

Beef SM	WB of calpain-1 in myofibrils; Increased fluorescence release after incubation of myofibrils with FITC labeled casein and Ca^{2+}; Increased desmin degradation after incubation of myofibrils with Ca^{2+}	Boehm et al. (1998)

LD *M. longissimus dorsi*; LT *M. longissimus thoracis*; LTL *M. longissimus thoracis et lumborum*; LM *M. longissimus lumborum*; PM *M. psoas major*; SM *M. semimembranosus*

WB western blot; WHC water-holding capacity; FITC fluorescein isothiocyanate

2.2. Early postmortem changes and proteolytic tenderization in meat

2.2.1. Early postmortem changes in meat

The most significant changes in muscle after slaughter include the decrease in pH and muscle temperature as well as the rigor formation and aging-induced effects on meat tenderness. Glycogen is metabolized by anaerobic glycolysis to supply ATP due to the failure of circulatory system to transport oxygen in muscles after slaughter. Lactate and protons are produced during this metabolic process and they cannot be transported out of the postmortem muscle and thereby the muscle pH declines. The rate and extent of postmortem pH decline are highly related to meat quality (Bee, Anderson, Lonergan, & Huff-Lonergan, 2007; Le Bihan-Duval et al., 2008; López-Bote, 2017). Additionally, muscle pH continuously interacts with temperature during rigor development and has a pronounced impact on proteolytic activity of proteases and protein denaturation, and consequently affect meat quality attributes (Hwang & Thompson, 2001; Kim et al., 2014). For example, the development of PSE muscle is attributed to the substantial protein denaturation and alteration of proteolytic potential of calpains as a result of high pre-rigor temperature and fast pH decline early postmortem (Kim et al., 2012; Liu et al., 2014; Offer & Knight, 1988). Except muscle pH, the extensibility of muscle is also greatly influenced by the postmortem metabolism of energy. The decline of pH and depletion of ATP cause dysfunction of sarcoplasmic reticulum and accelerated release of Ca^{2+} into the sarcoplasm and Ca^{2+} will not be pumped back into sarcoplasmic reticulum (Ji & Takahashi, 2006; Whiting, 1980). The concentration of Ca^{2+} in the sarcoplasm thus increases and further stimulates the contractile system. The myosin associates with the actin to form actomyosin cross-bridges and they can be broken down by ATP (Huff-Lonergan & Lonergan, 2005; López-Bote, 2017). However, the postmortem ATP provided by metabolism of glycogen is limited. Once ATP is exhausted, the muscle contraction is

permanent and thereafter the muscle enters the phase of rigor mortis. The tenderness of meat will increase after the tenderization process during postmortem storage.

2.2.2. Proteolytic tenderization of meat during postmortem storage

2.2.2.1. Calpain mediated meat tenderization

Meat tenderness is the one of most important eating quality characteristics (Shackelford et al., 2001). However, the inconsistency in meat tenderness caused by the large biological diversity of skeletal muscle affects its economic value (Sentandreu et al., 2002). Therefore, it is important to understand the tenderization process of meat during storage.

The tenderization process is mainly due to the structural and biochemical alterations at the level of myofibrils (Koohmaraie, 1996). Myofibrils are the most important structural component in skeletal muscle and occupy a very large (around 70%) volume in lean meat (Offer & Trinick, 1983). The structural network of myofibrils is built up by a large amount of proteins which comprise 60-70% of the total muscle proteins (Hamm, 1972). Myofibrillar proteins build up a flexible framework mainly assembled by thick and thin filaments. The thick and thin filaments are primarily made up of myosin and actin molecules, respectively (Purslow, 2017). Myosin molecule has a tail (light meromyosin), a collar (heavy meromyosin S-2) and a head region (heavy meromyosin S-1). The global head region of myosin contains the enzymatic ATPase catalytic site and the site for actin-binding (Rayment et al., 1993). The troponin complex is one of the major regulatory proteins that functions to regulate actin-myosin interaction and muscle contraction. Troponin is a calcium-dependent complex containing three subunits consisting of troponin I, troponin C and troponin T which have the functions of inhibiting actomyosin ATPase, binding Ca^{2+} and binding tropomyosin, respectively. Muscle contraction is regulated by Ca^{2+} concentration in the sarcoplasm via the troponin complex (Gordon, Homsher, & Regnier, 2000). Additionally, intermediate filaments play an important role in organizing the structure of the myofibrils. As shown by Fig. 2, desmin-containing intermediate filaments run between the myofibrils and are thereby linking adjacent myofibrils together. The filaments also extend and encircle the myofibrils to form a three-dimensional scaffold at the Z-disk. Furthermore, it connects the contractile apparatus to the subcellular organelles including nuclei and mitochondria, and to the specialized sites of adhesion-costameres in the sarcolemma (Capetanaki, Milner, & Weitzer, 1997; Koutakis et al., 2015; Milner, Mavroidis, Weisleder, & Capetanaki, 2000). Costameres are structures at the plasma membrane of skeletal muscle and they contribute to transmission of force from the

sarcomere to the sarcolemma and extracellular matrix, maintaining mechanical integrity of the sarcolemma, and orchestrating mechanically related signaling (Bloch et al., 2002; Peter, Cheng, Ross, Knowlton, & Chen, 2011).

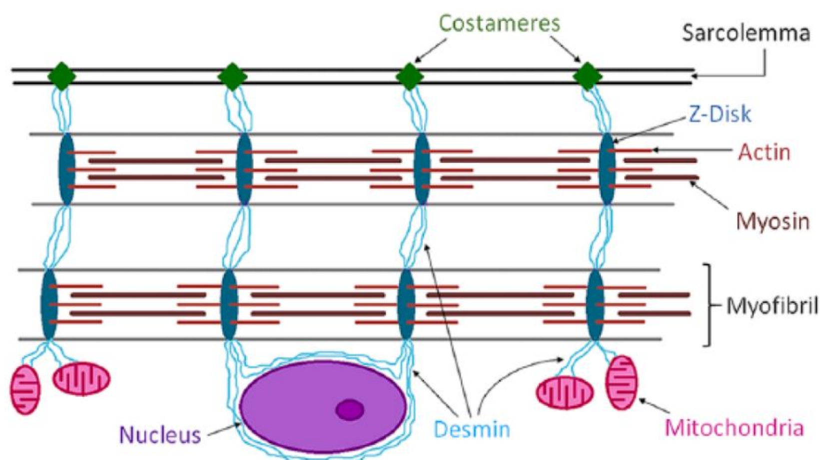

Fig. 2. Schematic diagram illustrating the association of intermediate filament desmin with myofibrils, sarcolemma, mitochondria and nucleus within a skeletal muscle fiber (Koutakis et al., 2015).

The calpain system is believed to be an important contributor to postmortem proteolysis and the resultant meat tenderization during storage (Bhat et al., 2018; Huff Lonergan et al., 2010; Taylor et al., 1995). As reviewed by (Goll et al., 2003), a large number of myofibrillar proteins that are important for maintaining the cytoskeletal structure within muscle fiber, such as demin, troponin T, titin, talin, filamin and nebulin, are substrates of calpains. Desmin degradation is accepted to contribute to meat tenderization due to the role of desmin in maintaining the structural integrity of the muscle fiber around the Z-line (Bhat et al., 2018; Huff-Lonergan et al., 1996; Pearce, Rosenvold, Andersen, & Hopkins, 2011). Proteolysis of troponin T may disrupt the integrity of the actin filaments and alter the interactions between actin and myosin, resulting in myofibril fragmentation (Bhat et al., 2018; Taylor et al., 1995). Calpains meet the criteria to be considered as a possible candidate to play a role in tenderization, as calpain-1 and calpain-2 are endogenous to skeletal muscle fiber, are able to reproduce postmortem proteolysis in myofibrils *in vitro* under optimum conditions and have access to myofibrils in muscle tissue (Goll, Otsuka, Nagainis, Shannon, Sathe, & Muguruma, 1983; Koohmaraie, 1996). Geesink, Kuchay, Chishti, & Koohmaraie (2006) argued that calpain-1 is largely responsible for postmortem proteolysis as a great inhibition of proteolysis in calpain-1 knockout mice was found

during 3-day storage as evidenced by limited degradation of proteins like desmin, troponin T and dystrophin. It has been well established that increased calpain activity leads to a higher degree of degradation of cytoskeletal proteins and also influences meat tenderization (Koohmaraie & Geesink, 2006). Injection of $CaCl_2$ into beef *longissimus lumborum* steaks has been reported to cause earlier activation of calpain-2 and thereby reduced shear force and increased sensory acceptance, and freezing of meat had similar but decreased effect on meat tenderness compared to Ca^{2+} injection (Colle et al., 2018). A similar effect of freezing on increase of free Ca^{2+} concentration and acceleration of calpain activation during early storage were found in pork LTL muscle (Zhang & Ertbjerg, 2018). Additionally, Dang, Buhler, Davis, Thornton, Scheffler, & Matarneh (2020) reported that calpain-1 activation was accelerated, and postmortem proteolysis and meat tenderness were enhanced by a greater Ca^{2+} concentration in sarcoplasm during aging after beef LTL muscle was injected with a cell-permeable inhibitor for the mitochondrial calcium uptake. Hopkins & Thompson (2001) found that injection of the cysteine protease inhibitor E-64 into lamb LTL muscle leads to limited meat tenderization by the observation of decreased myofibrillar fragmentation index and protein solubility and increased shear force, while a specific inhibitor for cathepsins B and L was also applied but no difference was found in these indicators, and thus they also argued that calpains play a pivotal role in postmortem proteolysis and meat tenderization. When ultrasonication was applied to bovine LTL (Dang, Stafford, Taylor, Buhler, Thornton, & Matarneh, 2022) and *semitendinosus* (Wang, Kang, Zhang, Zhang, Zou, & Zhou, 2018) muscles, then early activation of calpain-1, greater proteolysis as observed by increased degradation of desmin and troponin T, and lower shear force were found after storage, suggesting that increased calpain-1 activity induced by ultrasound treatment improves beef tenderness. It has been reported that oxidated cysteine residue in the active site of calpain-1 lead to loss of its activity (Lametsch, Lonergan, & Huff-Lonergan, 2008). Limited calpain-1 activation, reduced myofibrillar proteolysis and increased shear force were found in pork with high oxygen packaging (Chen, Zhou, & Zhang, 2015b) and in beef with irradiation (Rowe et al., 2004b). They thus suggested that oxidative conditions in meat will lead to inactivation of calpain-1 and thereafter decreased meat tenderization. In addition, S-nitrosylation of calpain-1 was suggested to cause limited activation of calpain-1 and reduced proteolysis of myofibrillar proteins in porcine LM muscle (Liu, Li, Wang, Zhou, & Zhang, 2016) and bovine SM muscle (Hou, Zhang, Zhang, Liu, Tang, & Zhou, 2020), and in parallel higher shear force was found in bovine SM muscle. Thus, they speculated that S-nitrosylation on calpain cysteine sites can decrease calpain activity and thereby meat tenderization (Hou et al., 2020). During

postmortem storage, calpain-1 activity decreased rapidly (Boehm et al., 1998; Colle & Doumit, 2017; Liu et al., 2014) while the degradation of cytoskeletal proteins increased and meat tenderness was improved (Huang & Forsberg, 1998; Lu, Zhang, Xu, Zhu, & Luo, 2020; Wang et al., 2018). Additionally, the activation of calpain-2 was found in meat with extended aging time in beef (Colle & Doumit, 2017), with high muscle temperature early postmortem in pork (Liu et al., 2014; Pomponio & Ertbjerg, 2012), with freezing-thawing process in pork (Zhang & Ertbjerg, 2018) and with post-rigor Ca^{2+} injection in beef (Colle et al., 2018). Therefore, calpain-1, and in some cases also calpain-2 are contributing to meat tenderization.

2.2.2.2. Other tenderness-related enzymes

The view that proteolytic tenderization is a multi-enzyme process including several enzyme systems such as calpain system, the proteasome, the lysosomal enzymes and the caspase system, has been gaining more support in recent years. It has been suggested that calpains initiate the weakening of the myofibrillar structures and thereafter the proteasome and cathepsins further degrade the remaining large fragments into small peptides (Ouali, 1990; Bhat et al., 2018; Calkins & Seideman, 1988; Houbak, Ertbjerg, & Therkildsen, 2008; Zeng et al., 2017). Cathepsins is a group of proteases present in lysosomes and was suggested by some researchers to involve in the meat tenderization (Ouali, 1990; Sentandreu et al., 2002). An increase in activity of cathepsin B + L and solubility of collagen, and reduction of shear force were found in bovine *semitendinosus* muscle after treatment with ultrasound (Wang, Li, Teng, Zhang, Purslow, & Zhang, 2022). They thus suggested that the increased activity of cathepsin B + L caused by ultrasound can be part of the reasons of meat tenderization. Zeng et al. (2017) observed desmin degradation after incubation of isolated myofibrils from pork with lysosomal enzymes. Baron et al. (2004) suggested that calpains lead to the initial cleavage of desmin and subsequently cathepsins further degrade the remaining desmin fragments which are resistant to calpain activity. However, the role of cathepsins in meat tenderness is still controversial because they are located in the lysosomal compartment and do not have access to the myofibrillar proteins at time of slaughter (Kaur, Hui, Morton, Kaur, Chian, & Boland, 2021). Proteasome (26S) is composed of a 19S subunit with regulatory activity and a 20S catalytic subunit and it can reversibly dissociate into the two subunits in the absence of ATP (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). The chymotrypsin-like activity of the proteasome during storage has been observed to decreased in pork (Zhang & Ertbjerg, 2018), ostrich *iliofibularis* muscle (Thomas, Gondoza, Hoffman, Oosthuizen, & Naudé, 2004)

and beef *Rectus abdominis* muscle (Lamare, Taylor, Farout, Briand, & Briand, 2002). Zeng et al. (2017) found release of α-actinin after isolated myofibrils were incubated with purified proteasome. Houbak et al. (2008) observed reduced degradation of structural proteins following inhibition of the proteasome chymotrypsin-like activity. Collectively, these reported results suggest a possible role of the proteasome in meat tenderization.

Meat tenderness has been reported to be enhanced by the mitochondrial apoptotic pathway during postmortem storage. Caspases are cysteine proteases related to apoptosis and able to cleave numerous proteins including myofibrillar proteins (Kemp & Parr, 2008; Wang, Han, Ma, Yu, & Zhao, 2017). It has been reported in spent hen after being treated with increased concentrations of adenosine 5′-monophosphate (AMP) that meat tenderization improved as observed by increased myofibril fragmentation index and lower shear force due to increased activity of cathepsin B and caspase 3 (Barido & Lee, 2021). This is likely attributed to the modulation of AMP on cell proliferation including muscle cells and greater occurrence of apoptosis induced by AMP binding onto specific γ-subregulatory sites of AMP kinase, and thereby contributing to meat tenderization during storage (Barido & Lee, 2021; Yang, Han, Yu, Gao, & Song, 2020). The relationship between meat tenderness and caspase activation has also been investigated by injection of Ca^{2+} and Zn^{2+} to meat (Huang, Ding, Zhang, Hu, Zhang, & Zhang, 2018). The activity of caspase reduced rapidly, and in parallel degradation of desmin, titin and nebulin, release of cytochrome c and meat tenderness (shear force decreased) became greater in beef steaks in the order Ca^{2+} > control > Zn^{2+}, suggesting that except the calpain system, caspases may also be a contributor to meat tenderization (Huang et al., 2018). In addition, Chen et al. (2011) and Kemp, Bardsley, & Parr (2006) suggested the role of caspases in postmortem proteolysis in chicken and pork tenderization, respectively. Zhang, Li, Yu, Han, & Ma (2019) reported that activity of cathepsin B and D increased in bovine *longissimus dorsi* muscle during aging and they can activate Bid and Bax in the mitochondria. Activated Bid and Bax triggered mitochondrial membrane permeability and further activated calpase-3 and caspase-9, and thereafter lead to apoptosis and meat tenderization. Interactions between the calpain system and the caspase system has also been reported (Kemp & Parr, 2012). It was reported that calpains can act as regulators for caspases (Chen, Feng, Zhang, Xu, & Zhou, 2012; Chua, Guo, & Li, 2000) and caspases can also modulate meat tenderization via its proteolysis on calpastatin (Chen, et al., 2015a; Huang, Huang, Zhang, Guo, Zhang, & Zhou, 2014; Kemp, King, Shackelford, Wheeler, & Koohmaraie, 2009; Wang, 2000). Collectively, the proteolytic tenderization process is increasingly becoming regarded as a

complex process including several enzyme systems and more studies are needed for a better understanding.

2.2.3. Role of postmortem proteolysis on water-holding

Reduction of water is another important change in meat. Water is the most abundant component in meat and accounts for around 75% of fresh lean meat (Offer & Trinick, 1983). Purge and drip losses are strongly related to economical and nutritional values of the meat. A large proportion of water in muscle is entrapped within the myofibrils, and the rest are often found in extra-myofibrillar spaces, such as between the myofibrils, between muscle fibers and between the muscle fiber bundles (Offer & Cousins, 1992). Water in meat is lost by surface evaporation during handling of carcasses and muscle cuts, and by the water exudation (drip loss or purge loss) (Offer et al., 1989). The water-holding of meat is influenced by factors such as rate and extent of pH decline, proteolysis and protein denaturation and oxidation (Huff-Lonergan & Lonergan, 2005; Kristensen & Purslow, 2001).

Structural proteins within the muscle fiber form a flexible framework, which are providing substantial connections between myofibrils, and between myofibrils and sarcolemma. The constraining forces produced by such structures have been suggested to limit the swelling of muscle fiber (Kristensen & Purslow, 2001). After slaughter, the shrinkage within the muscle fiber contributes to water migration from the intra- to extracellular space, and thereby leads to water loss (Kristensen & Purslow, 2001; Puolanne & Halonen, 2010). Postmortem proteolysis has been hypothesized by Kristensen & Purslow (2001) to relate to the improved WHC of meat during later storage (Fig. 3). During prolonged storage, degradation of cytoskeletal proteins could reduce the strength of cross-bridges that limits the swelling within the muscle fiber, leading to a loosened structure of the muscle fiber. Water would then diffuse from extra- to intracellular space of muscle fibers (Fig. 3C). An initial decrease followed by an increase of WHC have been observed in storage of pork (Wang, Yan, Liu, Fu, Zhou, & Zhang, 2016) and beef (Farouk, Mustafa, Wu, & Krsinic, 2012).

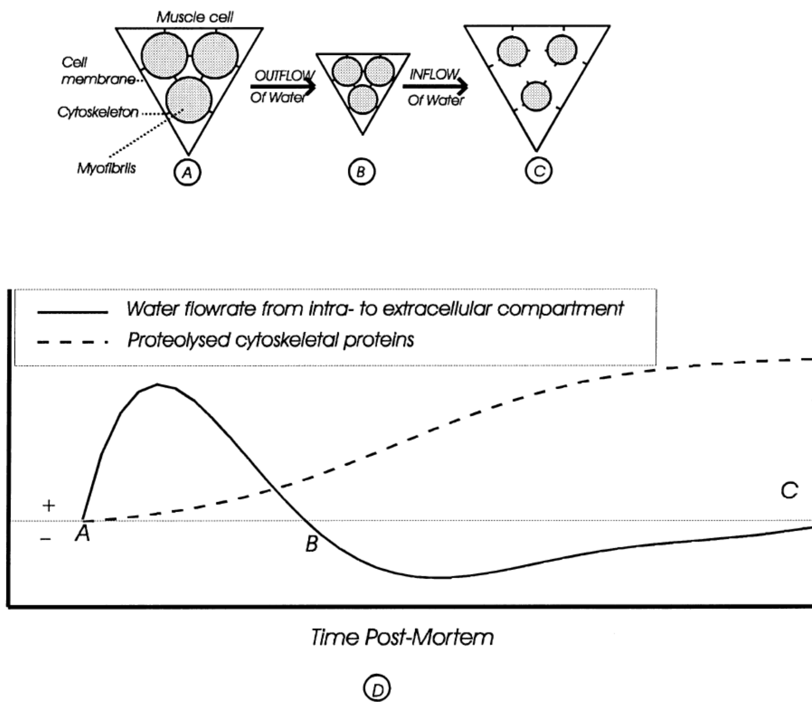

Fig. 3. Hypothesis for improved WHC as influenced by proteolysis. (A) simplified pre-rigor muscle fiber with three myofibrils connected to each other and to the sarcolemma. (B) Myofibril shrinkage leads to a shrinkage of whole muscle fiber, resulting in water flow from intra- to extracellular space. (C) Strain on the sarcolemma was removed by proteolysis of cytoskeleton, accompanied with water migration from extra- to intracellular space. (D) Relationship between water flow rate, postmortem time and degree of proteolysis (Kristensen & Purslow, 2001).

The volume change of myofibrillar lattice has been suggested to attribute to the reduction of WHC during meat storage (Hughes, Oiseth, Purslow, & Warner, 2014; Huff-Lonergan & Lonergan, 2005; Offer & Trinick, 1983). The transverse elements such as Z-disks can maintain the myofibril structure and thus limit the swelling or shrinking of myofibrils. Zeng et al. (2017) observed enhanced WHC of myofibrils when isolated myofibrils were incubated with calpain-2, proteasome and lysosomal extracts, and in parallel the degradation of desmin and α-actinin increased. They thus hypothesized that the swelling of myofibrils caused by degradation of proteins in and around Z-disks could improve WHC by allowing a water flow from outside to inside the myofibrils (Fig. 4). Similarly, improved WHC of myofibrils and degradation of proteins were also observed after the incubation of isolated myofibrils with sufficient Ca^{2+}, indicating there are enzymes bound to isolated myofibrils which could lead to

proteolysis of myofibrillar proteins in the presence of Ca^{2+} and thereby enhance WHC of myofibrils (Zeng et al., 2017, Study III).

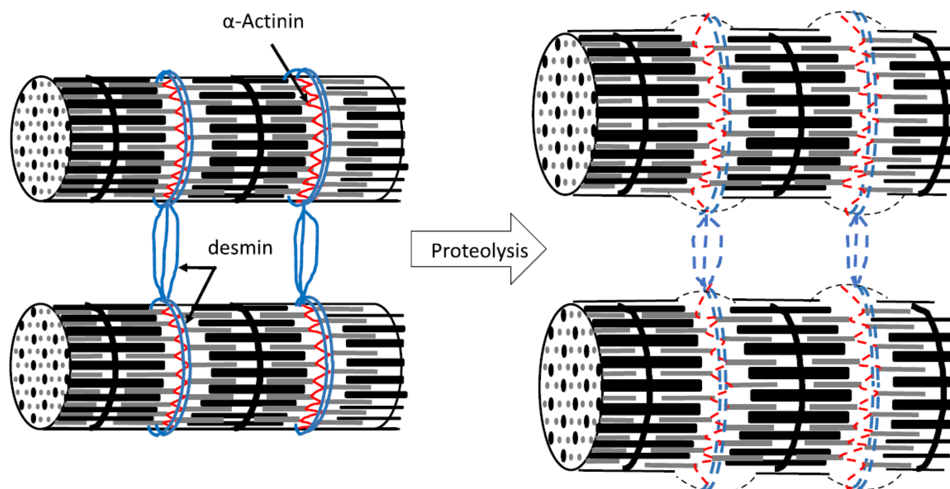

Fig. 4. Hypothesis illustrating structural changes within myofibrils caused by proteolysis of cytoskeletal proteins such as desmin and α-actinin (Zeng et al., 2017).

2.3. Calpain activity in meat and its influencing factors

2.3.1. Measurements for activity of calpains during storage

The content and activity of calpains highly influence the extent of proteolysis and thereby tenderization of meat. Changes on activity of calpains can be used as indicators for their involvement in meat tenderization. It is well known that the activity of calpains (especially calpain-1) and calpastatin in meat decreases during refrigerated storage (Dransfield, Etherington, & Taylor, 1992; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2001). The decline trend will be accelerated by higher muscle temperature postmortem (Boehm et al., 1998; Liu et al., 2014; Pomponio & Ertbjerg, 2012) and frozen-then-chilled storage (Zhang & Ertbjerg, 2018). Usually, the activity of calpains after their purification from muscle is measured by methods such as casein zymography and casein assay.

Casein assay

The casein assay is a convenient and quantitative method to determine the activity of calpains (Dayton, Goll, Zeece, Robson, & Reville, 1976). Casein is an inexpensive and readily available protein. It is a very good protein substrate for calpains that closely mimics the natural substrates and can be digested

at multiple sites rapidly and it thereafter produces short peptide fragments (Raser, Posner, & Wang, 1995; Thompson, Saldaña, Cong, & Goll, 2000). In standard casein assay, casein is used as the substrate of calpains, and the absorbance of trichloroacetic acid soluble peptides released from casein is measured at 278 nm. However, standard casein assay is not sensitive enough in some cases, and thus labeled casein has been used to improve the sensitivity (Veiseth et al., 2001). The labeling compounds such as fluorescent compounds FITC (Lonergan, Johnson, & Calkins, 1995; Twining, 1984), fluorescamine (Sogawa & Takahashi, 1978) and BODIPY-FL (Thompson et al., 2000) have been applied in the assay. Radiolabeled casein with ^{14}C-methylated (Delgado et al., 2001b; Veiseth et al., 2001) or ^{3}H (DeMartino, Huff, & Croall, 1986) has been used by measuring the radioactivity of degraded peptides with a greater sensitivity. Methods of ^{14}C and FITC labeled casein assay have been developed to determine the activity of myofibril-bound calpain-1 (Boehm et al., 1998; Delgado et al., 2001b).

Myofibrils as the substrate

In order to better reflect the proteolytic activity of calpains within meat structure, the substrate has been substituted from casein to isolated myofibrils in some studies (Study I) (Barrett, Goll, & Thompson, 1991; Dayton et al., 1976; Koohmaraie, Schollmeyer, & Dutson, 1986). In agreement with the casein assay, the increase of released peptides after incubation of myofibrils with calpains can be used to represent for the activity of calpains. In addition, the activity of calpains can be reflected as the extent of degradation of proteins in myofibrils such as desmin and troponin T via SDS-PAGE or western blot. Several studies have been conducted to estimate the capability of calpains to degrade myofibrillar proteins during aging by incubating myofibrils with calpains under conditions similar to postmortem storage (Geesink & Koohmaraie, 1999a; Kendall, Koohmaraie, Arbona, Williams, & Young, 1993; Koohmaraie et al., 1986; Mohrhauser, Underwood, & Weaver, 2011). After incubation of myofibrils with modified calpain-1, S-nitrosylation of calpain-1 has been reported to reduce its activity (Liu et al., 2016), while oxidative modification of calpain-1 can increase the activity (Qin, Deng, Lei, Liu, Lu, & Zhang, 2020). Half-maximal Ca^{2+} requirement for calpain-2 proteolysis of myofibrils has been observed to be 0.29 mM in study I. There was no significant difference in Ca^{2+} concentrations required for half-maximal proteolysis among casein, myofibrils and other protein- or peptide-substrates (Barrett et al., 1991).

Casein zymography

The activity of calpains in muscle is difficult to determine due to the presence of an excessive amount of their endogenous inhibitor calpastatin. Casein zymography is conducted on crude muscle extracts by only one centrifugation without the separation between calpains (calpain-1 and calpain-2) and calpastatin in advance, as the separation takes place in a gel during the electrophoresis. This approach can not only be used for the quantification of calpains, but also can reflect the autolysis condition of calpains and thereby it provides more information for the activity of calpains. Gelatin zymography has been used to detect and characterize metalloproteases (Milton, Norqvist, & Wolfwatz, 1992; Troeberg & Nagase, 2003), and then it has been applied to detect calpain activity as first descried by Raser et al. (1995). Samples are loaded and run in nondenaturing casein-containing polyacrylamide gels. Thereafter, the gels are incubated in a buffer containing Ca^{2+} to activate the calpains in the gel. After an appropriate time the reaction is stopped by addition of a buffer with EDTA. Casein in the region of an active calpain band is digested to small fragments which diffused out of the gel during incubation. Clear bands of calpains will appear after staining with Coomassie Brilliant Blue. Calpain-1 and calpain-2 and their autolyzed products can be easily separated due to different mobility on the gels (Raser et al., 1995). By casein zymography, Pomponio et al. (2008) have found four bands corresponded to native calpain-1, autolyzed calpain-1, native calpain-2 and autolyzed calpain-2 in pork after 3-day storage. Studies have focused on the changes of activity of calpains upon frozen then chilled storage (Zhang & Ertbjerg, 2018), high pre-rigor temperature incubation (Liu et al., 2014) and irradiation (Rowe et al., 2004b) during storage via casein zymography. Casein zymography has also been developed to distinguish the reversible and irreversible inhibitors by assessing the calpains activity on degrading casein (Raser et al., 1995).

Other methods

A simplified method based on the traditional casein assay has been developed. In this assay, the absorbance can be measured directly at 500 nm after the incubation of calpains and casein without any separation of the pellet and supernatant. It based on the turbidity of the suspension caused by the aggregation of hydrolysates during incubation (Jiang, Wang, Chang, & Chen, 1997). In the standard casein assay, the mixture needs to be well centrifuged after reaction as soon as possible. It is not

convenient and efficient when there is a large number of samples. This methodology has fewer steps in the protocol and is a continuous system which can be applied onto microplates (Jiang et al., 1997).

Gu, Whipple-VanPatter, O'Dwyer, & Zeece (2001) have applied capillary electrophoresis to measure the activity of calpains. This approach is very sensitive and fast and activity of 2-3 ng calpain can even be detected by using Oregon Green labeled β-casein as substrate.

2.3.2. Content and activity of myofibril-bound calpains

Generally, most of the measurements related to the activity of calpains in previous studies have been carried on the extracted calpains from muscle by the methods mentioned in 2.3.1. The extractable activity of calpains was used to reflect their involvement in meat tenderization. However, there are part of calpains binding to the subcellular organelles such as myofibrils, cell membrane and mitochondria as described in 2.1.1 and 2.1.3. The binding of calpains to the myofibrils is quite tight and cannot be easily removed or extracted. After 4 times washing of myofibrils, there was still myofibril-bound calpains and they were proteolytically active in the presence of sufficient Ca^{2+} (study I). Boehm et al. (1998) have reported that calpain-1 remained bound to myofibrils that have been washed 14 times including 2 times with the detergent triton X-100. Similarly, calpain-1 has been observed to still bind to myofibrils after 11 washes containing 2 triton-washes (Delgado et al., 2001b). In addition, several studies have reported that calpain-1 gradually bound to myofibrils during postmortem storage (Melody et al., 2004; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a) and they had some proteolytic activity (Boehm et al., 1998; Delgado et al., 2001b). It has been reported that limited calpain activity occurs in protein-denaturing conditions in meat as a result of high pre-rigor temperature in combination with fast pH decline (study III) and (Kim, Lonergan, & Huff-Lonergan, 2010; Kim et al., 2012). Thus, in meat upon high temperature incubation, the substantial decrease in extractable activity of calpains is likely due to the precipitation of calpains into myofibrillar fractions, rather than early activation as evidenced by faster decline of extractable calpain activity (Geesink, Bekhit, & Bickerstaffe, 2000; Hwang et al., 2004; Liu et al., 2014). Collectively, these studies show that a fraction of calpains are not in the sarcoplasm and cannot be measured as the extractable activity of calpains. Hence, assessing the proteolytic activity of calpains based only on their extractable activity will possibly underestimate the calpain level in postmortem muscle. Therefore, it

is not fully reliable to predict the involvement of calpains in meat tenderization only by the decrease of extractable calpain activity in the sarcoplasm.

It is difficult to determine the activity of myofibril-bound calpains. The amount of calpains, bound to myofibrils, is much smaller in relation to the amount of proteins in myofibrils. Only limited amount of myofibrillar proteins can be loaded onto a zymography gel and the proportion of calpains in loaded samples would be too small to be detected. Therefore, the above-described casein zymography are not available to study the activity of myofibril-bound calpains. Delgado et al. (2001b) determined the activity of myofibril-bound calpain-1 by incubation of the isolated myofibrils with ^{14}C labeled casein, and the measured activity of myofibril-bound calpain-1 against casein accounted for 4% of the total calpain-1 activity. The activity was very small considering the large amount of calpain-1 (around 40% of total calpain-1 as measured by dot-blot analysis) bound to myofibrils and thus it does not seem to be a good way to measure the activity myofibril-bound calpain-1. However, the myofibril-bound calpains showed more proteolytic activity when myofibrillar proteins instead of casein were used as the substrates. A few studies found that desmin (Boehm et al., 1998; Zeng et al., 2017), nebulin and titin were degraded to a large extent by myofibril-bound calpains (Delgado et al., 2001b). In Study III, we found degradation of desmin and troponin T, and decreased particle size by myofibril-bound calpains. Zeng et al. (2017) reported increased desmin degradation and peptides release in pork by myofibril-bound proteases which were suggested to be calpain-1 and calpain-2. It seems that the exogenous proteins cannot be hydrolyzed by myofibril-bound calpains possibly due to steric hindrance of access to the active sites in the myofibril-bound calpains. Calpastatin is one of the factors to regulate calpains activity, however, it cannot inhibit the proteolytic activity of myofibril-bound calpain-1 (Boehm et al., 1998; Delgado et al., 2001b). Similarly, it has been reported that mitochondrial associated calpain-1, particular in the intramembrane location, cannot be inhibited by calpastatin (Garcia et al., 2005).

2.3.3. Factors influencing calpain activity

Role of pre-rigor temperature and pH

Muscle temperature and pH change substantially after slaughter as described in 2.2.1. These changes significantly affect the extent and rate of activity of calpains and consequently influence meat tenderization (Kim et al., 2014).

Pre-rigor muscle temperature and pH are negatively linked and thus a high pre-rigor temperature will cause a faster drop of pH. It is still unclear whether a fast or slow pH decline postmortem improves the meat tenderization. Several studies have found that high pre-rigor temperature combined with a faster pH decline, which tend to induce the development of PSE meat, can earlier activate calpains and lead to tender meat (Geesink et al., 2000; Hwang et al., 2004; Liu et al., 2014; Pomponio & Ertbjerg, 2012). In the current thesis it was found that faster translocation of calpains from the sarcoplasm to myofibrils and higher degree of proteolysis on myofibrillar proteins in muscle occurs upon pre-rigor incubation at 22 and 30 °C than that of 14 °C (study III). The earlier activation and translocation of calpains are presumably due to the increased Ca^{2+} concentration induced by the high pre-rigor temperature (study III, Hwang et al., 2004). However, it has been reported that the early activation of calpains and thereby tenderness improvement caused by high pre-rigor temperature was only a transient effect, and no further tenderization took place during longer storage due to the early exhaustion of proteases (Dransfeld, 1994; Kim et al., 2014).

In contrast, it has been reported that faster pH drop led to higher degree of protein denaturation and thereby limited activity of calpain-1 and calpain-2 (Claeys, De Smet, Demeyer, Geers, & Buys, 2001; Kim et al., 2010; Kim et al., 2012). The presence of incompletely autolyzed calpain-1 after relative long storage was suggested to be attributed to the protein-denaturing condition as a result of high pre-rigor temperature (Kim et al., 2010). After a period of storage, incompletely autolyzed calpain-1 was observed in muscle under higher pre-rigor temperature, while only completely autolyzed calpain-1 in muscle with relatively lower pre-rigor temperature was found in study III and by Kim et al. (2010). These results may suggest that high muscle temperature and fast pH decline early postmortem lead to precipitation of calpain-1 onto myofibrils and thereby limited proteolytic activity during aging. These contrasting effects of temperature and pH on activity of calpains may depend on the degree of high temperature: moderately high pre-rigor temperature (14 - 30 °C, Study III) in combination with moderate rate of pH decline may increase the concentration of Ca^{2+} and then lead to earlier activation

and translocation of calpains and thereby tenderness improvement, while too high temperature (> 38 °C, Study III) with very fast pH drop will limit calpain activity and the extent of tenderization.

Role of oxidation and nitrosylation modification

Protein oxidation has been known as one of the major factors that influence meat quality. Calpains are cysteine proteases and the cysteine and histidine residues in calpains are susceptible to oxidation and subsequently inhibit calpain activity and lead to limited postmortem proteolysis and meat tenderization (Bao & Ertbjerg, 2019; Bhat et al., 2018; Lametsch, Lonergan, & Huff-Lonergan, 2008). Several studies have reported that calpain oxidation resulted in loss of activity. The activity and autolysis of calpain-1 reduced in beef incubated with H_2O_2 saline solution (Ding, Wei, Zhang, Zhang, & Huang, 2021) and irradiated beef samples (Rowe et al., 2004b). Similarly, decreased activity and autolysis of calpain-1 was observed in beef (Fu, Ge, Liu, Wang, Zhou, & Zhang, 2017) and pork (Chen, Zhou, et al., 2015) with high oxygen modified atmosphere packaging. However, increased degradation of myofibrillar proteins by oxidized calpain-1 was observed upon oxidation of calpain-1 with moderate concentration of H_2O_2 (0.5 mM), and it was attributed to the changed secondary and tertiary structure of calpain-1 by the observation of increased carbonyl and decreased sulfhydryl content of calpain-1, (Liu et al., 2021). Bao & Ertbjerg (2019) suggested that the proteolysis is influenced by the degrees of oxidation. Moderate oxidation improves proteolysis by unfolding protein structures, while more compact protein structures as a result of extensive oxidation lead to decreased proteolysis.

In addition, oxidative modification of myofibrillar proteins affect their susceptibility to proteolysis. Fu, Liu, Zhang, Ben, & Wang (2020) reported that degradation of myosin by calpain-1 enhanced after myosin was incubated with 0–100 μM H_2O_2 which was attributed to increased exposure of the oxidation sites of myosin. Degradation increased for actin but decreased for myosin heavy chain, troponin T, and desmin after isolated myofibrils were exposed to increased H_2O_2 concentration and thereafter incubated with calpain-1 (Zhang et al., 2021c). Chen, Huang, Huang, Huang, & Zhou (2014) reported that degradation of isolated desmin from bovine muscle decreased with increased concentration of H_2O_2 and Fe^{2+}. These contrasting results may due to that oxidative modification of myofibrillar proteins induce various susceptibility to proteolysis.

As reviewed by Liu, Warner, Zhou, & Zhang (2018), nitric oxide (NO) and protein S-nitrosylation may contribute to the process of muscle to meat conversion through the regulation of glycolysis, Ca^{2+}

release, proteolysis and apoptosis. NO is produced in skeletal muscle and act as a signaling molecule (Pacher, Beckman, & Liaudet, 2007). NO could react with the protein sulfhydryl to form S-nitrosothiol (protein S-nitrosylation) which is able to change the protein activity, stability and interaction (Foster, Hess, & Stamler, 2009; Li et al., 2021). It was reported that S-nitrosylation of calpain-1 on the cysteine sites lead to limited autolysis of calpain-1 and reduced proteolysis of myofibrillar proteins in pork (Li, Liu, Zhang, Fu, Liu, & Zhou, 2014; Liu et al., 2016; Liu, Lonergan, Steadham, Zhou, Zhang, & Huff-Lonergan, 2019a) and beef (Hou et al., 2020), and in parallel higher shear force was found in bovine SM muscle, thus they speculated that protein S-nitrosylation can also influence calpain-1 activity and thereby meat tenderization (Hou et al., 2020). Zhang, Liu, Wang, Kang, Zhou, & Zhang (2018) studied the effect of NO on calpain activation by incubation of beef with NO donor and synthase inhibitor for 24 h at 4 °C and subsequently aging for 7 days at 4 °C. They found that calpain-1 autolysis and degradation of desmin and troponin T increased by NO synthase inhibitor while decreased by NO donor, and thus they suggest that NO could influence meat tenderization via regulation of calpain activation and proteolysis activity. In addition, S-nitrosylation of myofibrillar proteins affects their proteolytic susceptibility. It has been reported that degradation of desmin and titin increased, while troponin T degradation reduced by calpain-1 after S-nitrosylation of isolated myofibrils, and it was suggested to be caused by changed redox state of myofibrillar proteins (Liu, Lonergan, Steadham, Zhou, Zhang, & Huff-Lonergan, 2019b).

2.4. Hypothesis for meat tenderization caused by myofibril-bound calpains

2.4.1. Current explanation

The tenderization process during storage is complex and currently not fully understood. It has been generally accepted that the proteolysis of key myofibrillar proteins by calpains and other proteolytic enzymes as described in section 2.2.2 is the principle reason for meat tenderization (Huff Lonergan et al., 2010; Lana & Zolla, 2016). The calpain system is believed to play an important role in this process (Koohmaraie, 1994). In addition to enzymatic mechanisms, high ionic strength was supposed to contribute to the weakening of myofibrils as reviewed by Ouali (1990). A non-enzymatic mechanism has been proposed to contribute to the meat tenderization. The author suggested a direct effect of Ca^{2+} on myofibrillar proteins and connective tissue proteins, and this effect is pH dependent (Takahashi, 1992; Takahashi, 1996). However, this hypothesis is not widely accepted.

2.4.2. Mechanisms of myofibril-bound calpains on meat tenderization

It has been widely accepted that calpain system, especially calpain-1 plays a significant role in meat tenderization. However, the mechanism of how calpains involve in meat tenderization has not been well understood so far. Furthermore, the contribution of calpain-1 to tenderization has been questioned mainly due to the fact that no direct relationship has been found between aging rate and the level of calpain-1 during postmortem storage (Boehm et al., 1998; Ji & Takahashi, 2006; Ouali, 1990).

Calpains may work on the degradation of myofibrillar proteins by the form of myofibril-bound calpains. The level of free Ca^{2+} in muscle increases during postmortem storage (Hwang et al., 2004; Ji & Takahashi, 2006; Zhang & Ertbjerg, 2018). Calpains are activated by the increased Ca^{2+} concentration, and thereafter the activated calpains gradually bind to subcellular organelles such as myofibrils (Study II and III) (Boehm et al., 1998; Delgado et al., 2001b; Rowe et al., 2004b). It has been found that Ca^{2+} can induce purified calpain-2 to become tightly bound to isolated myofibrils and that the myofibril-bound calpain-2 was proteolytically active and able to degrade desmin in the presence of Ca^{2+} (Study I). Once myofibril-bound calpains complete the degradation of available proteins in the vicinity of where it is bound in the myofibril structure, one possibility is that the calpains will be released from the myofibrils to the sarcoplasm. In agreement, the content and activity of myofibril-bound calpain-1 were observed to increase early postmortem and thereafter decrease during later storage (Study II) (Delgado et al., 2001b). The amount of sarcoplasmic calpain-1 decreased slower later postmortem than early postmortem (Study II). This may be due to the release of calpain-1 from myofibrils back to the sarcoplasm after proteolysis. The translocation of calpains and their proteolytic activity are regulated by the concentration of Ca^{2+} in the sarcoplasm. A higher concentration of Ca^{2+} in the early period of postmortem storage has been found in muscle upon pre-rigor incubation with higher temperature (Study III, Hwang et al., 2004). Faster translocation of calpain-1 and more degradation of desmin early postmortem which was probably caused by increased Ca^{2+} concentration in the meat incubated at higher pre-rigor temperature were observed in study III. More purified calpain-2 bound to isolated myofibrils with higher Ca^{2+} concentration and thereby more proteolysis by myofibril-bound clapain-2 were found in study I. In addition, it has been reported that calpastatin cannot inhibit the activity of calpains once calpains bound to myofibrils or mitochondria (Delgado et al., 2001b; Garcia et al., 2005). The myofibril-bound calpains may provide an explanation for why calpains, especially calpain-1, are able to degrade myofibrillar proteins in the presence of an excess amount of calpastatin. The reported

influence of calpastatin on meat tenderness may thus be due to its influence on the amount of calpains becoming bound to myofibrils (Study II).

3. Objectives

The aim of this thesis was to investigate the translocation of calpains in meat during postmortem storage and the proteolytic activity of myofibril-bound calpains in relation to the myofibrillar protein degradation and meat tenderization. To achieve this, the role of Ca^{2+} in the binding of purified calpains to isolated myofibrils and activity of myofibril-bound calpains was studied. Additionally, refrigerated storage, and high pre-rigor temperature incubation followed by refrigerated storage were applied to investigate the translocation of calpains and activity of myofibril-bound calpains during pork storage. The specific aims were:

- To investigate the role of Ca^{2+} in the process of calpain-2 binding to myofibrils and the proteolytic potential of myofibril-bound calpain-2 to degrade myofibrillar proteins (Study I)
- To investigate the distribution of calpain-1 and calpain-2 between sarcoplasmic and myofibrillar fractions and the proteolytic activity of myofibril-bound calpains during 12 days refrigerated storage of porcine LT muscle, and to provide new insight on myofibril-bound calpains induced protein degradation (Study II)
- To investigate the effect of pre-rigor temperature incubation (14, 22, 30 and 38 °C) on protein denaturation, Ca^{2+} induced calpain translocation and proteolytic activity of myofibril-bound calpains in relation to myofibrillar protein degradation and tenderness of pork LT muscle (Study III)

4. Materials and methods

A brief summary of the materials and methods used in the current thesis is described in this chapter, and more details can be found in the attached publications (study I, II & III).

4.1. Sample processing

Muscle

Pigs (in Study I and II, age160–165 days, the cross Norwegian Landrace × Swedish Yorkshire × Danish Landrace; in Study III, the cross Landrace × Yorkshire × duroc, the carcass average weight 88.66 Kg) were stunned by CO_2 and slaughtered in a local slaughterhouse in Finland (HKScan Slaughterhouse, Forssa). The visible connective tissue as well as external fat were trimmed off the LT muscles. The pork loins for the different studies were collected from pigs slaughtered between 2018 and 2020.

Sampling

Five pork loins were vacuum-packaged then transported in a container with some ice to the meat laboratory in University of Helsinki at 10 h postmortem in Study I and II, respectively. In Study I, meat samples (120–150 g) were cut from each LT muscle and they were further divided into several small proportion (around 15 g each). Muscle samples were then vacuum packaged at 12 h postmortem and frozen at -80 °C for further analysis. Of the five loins, two muscles were used for calpain-2 purification and the other three pieces were used for preparation of isolated myofibrils. DEAE Sepharose Fast Flow column, salting out, Sephacryl S-300 column, RESOURCE Q column were used in the purification work. Activity of calpain-2 after different purification steps is shown in Table 2.

Table 2. Activity of calpain-2 after different purification steps.

Purification steps	Protein (mg/ml)	Volume (ml)	Activity (U/ml)	Total activity (U)	Activity/Protein (U/mg)
DEAE fast flow	0.1	224	2.8	627.2	6.5
Salting out	5.3	8	108.5	868.0	13.1
Sephacryl S-300	0.8	54	9.0	486.0	11.8
RESOURCE Q	3.1	4.3	68.7	295.4	21.9

In study II, around 400 g muscle samples from pork loins of five different animals were removed and cut into small pieces (about 2 cm × 2 cm × 1 cm) and then vacuum-packaged followed by stored at 2 ± 1 °C in a cold room for 1, 3, 6, 9 and 12 days. Each animal was represented at every storage period. After storage, all muscle samples were frozen at -80 °C for further use.

Five LT muscles were obtained from five different pig loins in Study III and excised around 1 h post-mortem. A small part (around 40 g) from each muscle was collected and frozen in liquid nitrogen, being used as the reference samples at 1 h postmortem for further analysis. Each of the muscle was separated into four pieces and each of the piece was further divided into two parts. Muscle cuts were sealed in polyethylene bags and incubated in water bath at 14, 22, 30 and 38 °C from approximately 1.5 h postmortem until 6 h postmortem, and thereafter all muscle pieces were incubated in 14 °C water bath for another 2 h and stored at 2 ± 1 °C for 1 and 4 days. After chilled storage, all muscle samples were frozen at -80 °C until analysis. Muscle temperature and pH were determined before incubation (1 h postmortem). Additionally, measurement of pH was conducted at 2, 4, 6, 8 and 30 h postmortem.

4.2. Physical analysis

The determination of pH and temperature

Ultimate pH (24 h postmortem) for muscle samples in Study I and II was measured via inserting a pH electrode (Mettler-Toledo Inlab 427) to the center of meat tissue. In Study III, pH was measured from homogenates by homogenizing meat (around 0.5 g) with 10 volumes of 5 mM sodium iodoacetate and 150 mM KCl and then determined by pH electrode. For each of the five muscles, duplicates were done for 1 h-pHs and triplicates were performed for pHs at other storage times.

Purge loss and cooking loss

The weight of muscle after treatment (pre-rigor incubation with increased temperature) was taken as the initial weight. Muscle was slightly blotted dry and then weighed after each storage time. Purge loss (Study III) was determined by taking the weight difference of meat before and after storage and expressed as percentage loss of initial weight. For the measurement of cooking loss (Study III), the meat was vacuum packaged and cooked in 72 °C water bath for 60 min followed by cooling in cold water for 30 min. Cooking loss was calculated by the weight variations before and after cooking (expressed as percentage loss). Total loss (Study III) was defined as the weight difference between the

initial weight and the weight after cooking, and expressed as percentage loss of initial weight. For each temperature, 5 replicates were done for purge loss and cook loss.

Allo-Kramer shear force

Allo-Kramer shear force (Study III) was determined as described by Liu et al. (2014). Pork samples were cooked in 72 °C water bath for 60 min and then cooled in cold water for 30 min. The samples were cut into 8-10 small pieces of 20 × 20 × 6 mm (fiber along the 20 mm direction) and the Allo-Kramer shear cell was applied for the determination of shear force. For each of the five muscles, 8-10 repeats were done for shear force.

WHC of myofibrils

WHC of myofibrils was measured based on a centrifugation method described by Zhang & Ertbjerg (2018). Myofibril pellet was washed by MES buffer (75 mM KCl, 100 mM MES, 2 mM $MgCl_2$, pH 5.4) followed by a 10 min centrifugation at 2,400×g, and then the myofibril pellet was put in a 100 °C oven overnight. WHC of myofibrils was calculated the variations of water remaining in myofibrils by the weight difference of myofibril pellet before and after drying. Three independent batches were prepared, and all five LT muscles were pooled in each batch. For each batch, 12 replicates were performed for WHC, resulting in 36 replicates for each determination.

Free Ca^{2+} concentration

Concentration of free Ca^{2+} was measured at room temperature by a Ca^{2+} ion selective electrode (perfectION™ Combination Ca^{2+} Electrode, Mettler Toledo AG, Greifensee, Switzerland) based on a method of Ca^{2+} standard addition described by Zhang & Ertbjerg (2018). For free Ca^{2+} in myofibril suspension (15 mg myofibrillar protein/mL), the suspension was added with various concentrations of Ca^{2+} then mixed before measurement (Study I). For free Ca^{2+} in the sarcoplasm, meat samples (20 g) were finely chopped and centrifuged to collect the supernatant (Study III). The samples was added with 4 M KCl (50 : 1) for internal adjustment. Relationship between Ca^{2+} concentration and millivolts were established by a calibration curve. For each of the five muscles, duplicates were done for Ca^{2+} determination.

Particle size

Particle size of myofibril suspensions (Study III) was determined by a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK). The refractive index was set to 1.46 and the absorption coefficient to 0.01, and the particles were considered as nonspherical. Five repeated analysis were applied to each of the myofibril suspension by using distilled water as dispersant. D(v,0.1), D(v,0.5) and D(v,0.9) are used to describe the particle size for which 10%, 50% and 90% of the sample is below the corresponding size, respectively. D(3,2) and D(4,3) represent the mean diameter in surface area and volume, respectively. Two independent batches were prepared, and all five LT muscles were pooled in each batch. For each batch, 5 incubation replicates were performed for particle size, resulting in 10 replicates for each determination.

4.3. Biochemical analysis

Activity of purified calpain-2

Activity of purified calpain-2 was measured by using casein (Study I) and myofibrillar proteins (Study I and II) as substrate. Purified calpain-2 was incubated with casein and 5 mM Ca^{2+} for 30 min at 25 °C then stopped by 10% trichloroacetic acid followed by a centrifugation at 20,000 ×g for 5 min. Absorbance of the supernatant was measured at 278 nm. The increase of absorbance at 278 nm of 1.0 per hour was defined as one unit of calpain activity.

Purified calpain-2 was incubated with isolated myofibrils (15 mg myofibrillar protein /mL) and 5 mM Ca^{2+} at 25 °C for 2 h. The reaction was stopped by EGTA and the mixture was centrifuged at 20,000 ×g for 5 min to collect supernatant. Absorbance for the collected supernatant was read at 278 nm. The activity of calpain-2 on myofibrils was calculated by the absorbance difference of the sample with or without addition of calpain-2.

Activity of myofibril-bound protease

In order to measure the proteolytic activity of myofibril-bound proteases within myofibrils, the isolated myofibrils were prepared from meat samples at 12 h postmortem for Study I, after each storage time for Study II, after treatment (pre-rigor incubation with high temperature) followed by refrigerated storage for Study III.

The proteolytic activity of myofibril-bound calpain-2 was measured following the binding process of purified calpain-2 to isolated myofibrils (Study I). Isolated myofibrils were mixed with purified calpain-2 and then different amount of Ca^{2+} (0 mM Ca^{2+} was substitute by EGTA) was added into the mixture to induce the binding of calpain-2 to the isolated myofibrils. The myofibril pellet was then immediately washed to remove the unbound calpain-2. Thereafter, the myofibril pellet was kept on ice until measuring the proteolytic activity of myofibril-bound calpain-2.

The isolated myofibril suspension was incubated with different amount of Ca^{2+} at 25 °C for 2 h. The reaction was stopped by EGTA. The supernatant was collected after 8 min centrifugation at 20,000×g and the absorbance was determined at 278 nm to reflect the amount of released peptides (Study II). All myofibril pellets were put on ice before being processed for western blot followed by quantification of the desmin (Study I, II and III) and troponin T (Study III), and particle size (Study III). It should be noticed that the incubation of isolated myofibrils with Ca^{2+} in the current thesis was conducted at the optimum pH for calpains in order to better reflect the proteolytic potential of myofibril-bound calpains.

SDS-PAGE and western blot

Myofibril suspension (Study I, II and III) and meat sarcoplasm (Study II and III) was prepared for SDS-PAGE analysis. Protein concentration of samples was measured and adjusted by DC Protein Assay Kit. NuPAGE™ LDS Sample Buffer (4 ×) and NuPAGE™ Sample Reducing Agent (10 ×) (Invitrogen, Carlsbad, CA) were added and mixed with diluted protein samples followed by heated at 70 °C for 10 min, and then loaded onto NuPAGE™ Novex 12% Bis-Tris gels (desmin and troponin T) and 4-12% Bis-Tris gels (calpain-1 and calpain-2). The electrophoresis was performed at 200 V for approximate 50 min.

After electrophoresis, proteins in SDS-PAGE gels were transferred at 30 V for 1 h for desmin (Study I, II and III) and troponin T (Study III), and 12 h for calpains (Study II and III). After blotting, membranes were blocked for 1 h at 25 °C with skim milk powder in TBS (50 mM Tris, 150 mM NaCl, pH 7.5). Thereafter, membranes were incubated with primary antibodies for calpain-1 (MA3-940 Invitrogen, Carlsbad, CA) (Study II and III), calpain-2 (ab39165 abcam, Cambridge, UK) (Study II), and desmin (DE-U-10 Invitrogen, Carlsbad, CA) (Study I and II) at a dilution of 1:1000 in TBST (50 mM Tris, 150 mM NaCl, 0.5 g/L Tween-20, pH 7.5), for troponin T (T6277 Sigma, St. Louis, MO, USA) and desmin (D8281 Sigma, St. Louis, MO, USA) in study III at a dilution of 1:15000 and 1:3000

in TBST, respectively. After probing, membranes were washed and scanned then quantified by Odyssey Infrared Imaging System-CLx (LI-Cor Cop, Lincoln, NE) as described by Zeng et al. (2017). The fluorescence intensity of each band was calculated based on the intensity divided by the mean value of the standard and were expressed as relative intensity (%). For sarcoplasmic calpains, the reference standard with the same content as other samples at different storage time was taken as 100%; for the myofibril-bound calpains, only 25% of the reference standard (compared to sarcoplasmic samples) was loaded due to the lower content of calpain-1 in the myofibril fraction. The content of myofibril-bound calpain-1 was corrected to be comparable to the muscle content of sarcoplasmic calpain-1 using a myofibril : sarcoplasm protein ratio of 2:1. For each of the five muscles, duplicates were made for the sarcoplasmic and myofibril calpains in Study II and III. Three independent batches were prepared, and all five LT muscles were pooled in each batch. For each batch, two incubation repeats were done for desmin in Study II, 4 replicates were performed for desmin and troponin T in Study III. For each muscle, duplicates were conducted for desmin, and a representative western blot image was shown and the results were replicated in Study I.

4.4. Statistical analysis

Data analysis was performed using general linear model in the IBM SPSS Statistics 25 software. The significant differences between mean values were evaluated by the Bonferroni test with a level at $P < 0.05$. The animal number was set as random factor. Refrigerated storage time (Study II & III) and treatment of pre-rigor incubation with increased temperature (Study III) were set as fixed factor.

5. Summary of results

5.1. Changes in free Ca^{2+} concentration during storage and its role in the binding of calpain-2 to myofibrils

Changes in concentration of free Ca^{2+}

Pre-rigor muscle was incubated at increased temperature (14, 22, 30 and 38 °C) then stored at 2 °C for 4 days. The level of free Ca^{2+} in sarcoplasm increased with postmortem storage and higher pre-rigor temperature of muscle ($P < 0.01$). The initial concentration of free Ca^{2+} in muscle incubated at 14 °C was 50 µM (8 h postmortem) and it significantly increased to around 170 µM on day 4. However, a much higher ($P < 0.05$) initial Ca^{2+} concentration (230 µM) was found in muscle incubated at 38 °C and it gradually increased to 360 µM after 4 days of chilled storage (Tables 1 and 2, Study III).

Effect of free Ca^{2+} on activity of calpain-2 and binding of calpain-2 to isolated myofibrils

Proteolytic activity of calpain-2 increased after isolated myofibrils and purified calpain-2 were incubated with increased Ca^{2+} concentration (Fig. 3A, Study I). A faster increase ($P < 0.05$) of the activity with free Ca^{2+} concentration up to 0.9 mM, and thereafter a slower increase ($P > 0.05$) to the maximum value were observed. Although this is a Ca^{2+} level much greater than the reported Ca^{2+} concentration in the sarcoplasm of muscle during storage, it does come into range of the ionized level in serum (1.3 mM as typically found). The influence of free Ca^{2+} on the ratio of purified calpain-2 being associated to isolated myofibrils demonstrated a similar increasing trend in comparison with that of calpain-2 activity (Fig. 3B, Study I). The half-maximal Ca^{2+} requirements for the binding of calpain-2 to isolated myofibrils and hydrolysis activity of calpain-2 against myofibrils were 0.60 mM and 0.29 mM, respectively (Fig. 3, Study I).

Desmin degradation was chosen to be an indicator for proteolytic activity of myofibril-bound enzymes. Desmin degradation products gradually accumulated after incubation of isolated myofibrils with Ca^{2+} (Fig. 4A, Study I), showing that there was endogenous Ca^{2+}-activated enzyme in the isolated myofibrils. Isolated myofibrils were mixed with purified calpain-2 then briefly exposed to Ca^{2+}, followed by washing off unbound calpain-2, then re-incubated the myofibrils with Ca^{2+} and extensive desmin degradation was observed (Fig. 4C, Study I). Isolated myofibrils mixed with calpain-2 but not shortly exposed to Ca^{2+} (substituted with EGTA) before washing and re-incubation did not produce further

degradation of desmin (Fig. 4B, Study I). After binding of calpain-2 to isolated myofibrils with increased concentrations of free Ca^{2+}, then washing and re-incubation the myofibrils with specific Ca^{2+} concentrations, desmin degradation started to be observed at 0.1 mM Ca^{2+}, and it gradually increased with the increase of free Ca^{2+} level.

Stability of myofibril-bound calpain-2

The effect of washing on the stability of myofibril-bound calpain-2 is illustrated in Fig. 6 (Study I). The amount of desmin degradation products decreased gradually with increased number of washing-times. All intact desmin was degraded by the myofibril-bound calpain-2 after one time washing for the myofibrils. There was still sufficient active myofibril-bound calpain-2 to partly degrade desmin even after 4 times washings.

5.2. Translocation of calpains and changes of activity of myofibril-bound calpains during storage

Translocation of calpains

During the 12-day storage of pork at 2 °C, native calpain-1 was found in sarcoplasm only at 12 h and day 1, and there was only autolyzed calpain-1 in the rest of the storage (Fig. 1, Study II). For the pork pre-rigor incubated with increased temperature followed by 4-day storage at 2 °C, native calpain-1 was observed in both the sarcoplasm and myofibrils on day 1. On day 4, there was only autolyzed calpain-1 in both the fractions of muscle at 14, 22 and 30 °C, while native calpain-1 was still present in the sarcoplasm and myofibrils of muscle incubated at 38 °C (Fig. 2, Study III).

In Study II, the amount of sarcoplasmic calpain-1 on day 1 was 71% of that at 12 h, and thereafter it rapidly decreased ($P < 0.05$) to 45% on day 6 followed by a slower decrease ($P > 0.05$) until day 12 (30%). The content of calpain-2 in sarcoplasm showed a parallel but slightly slower declining trend than that of calpain-1. However, the amount of myofibril-bound calpain-1 increased with storage until day 6 and thereafter decreased. The maximum amount of myofibril-bound calpain-1 at day 6 was 17% of 12 h-sarcoplasmic calpain-1, which was significantly higher compared to other days (Figs. 1 and 2, Study II). The pre-rigor temperature of muscles affected the content of calpains in the sarcoplasm and myofibrils ($P < 0.01$) (Table 1, Study III). The amount of sarcoplasmic calpain-1 decreased with the

increase of incubation temperature, and it was significantly higher in meat incubated at 14 and 22 °C than in that of 30 and 38 °C on both days. In meat incubated at 14, 22 and 30 °C, the amount of calpain-1 in the sarcoplasm on day 4 was higher than that of day 1, while the content of myofibril-bound calpain-1 decreased on day 4, which could be due to higher pre-rigor temperature accelerated the translocation of calpain-1 from sarcoplasm to myofibrils and the amount of myofibril-bound calpain-1 reached its maximal point before day 4 and myofibril-bound calpain-1 started substantially releasing back to sarcoplasm. However, in meat incubated at 38 °C, the amount of sarcoplasmic and myofibril-bound calpain-1 did not change from day 1 to day 4. The content of myofibril-bound clapain-1 increased with higher temperature ($P < 0.01$), and it gradually decreased ($P > 0.05$) with storage in meat incubated at 14, 22 and 30 °C, while no evident change in content of myofibril-bound calpain-1 during storage was observed in muscle incubated at 38 °C ($P > 0.05$). At day 1, the content of myofibril-bound calpain-1 in muscle at 30 °C was 24% of 1 h-sarcoplasmic calpain-1 which is significantly lower than that of 38 °C (52%), while significantly higher compared to 14 °C (15%). A similar trend for myofibril-bound calpain-1 was observed in meat at day 4 (Fig. 2, Study III).

Activity of myofibril-bound calpains

Activity of calpains during postmortem storage has been reported to be associated to the tenderization of meat. Desmin is one of the most susceptible substrates of calpains and its degradation can reflect activity of calpains to some extent. The content of intact desmin decreased significantly in meat during refrigerated storage, while almost no desmin was degraded on day 1 (Fig. 3, Study II). Myofibrils were isolated and then incubated with Ca^{2+} to demonstrate the activity of myofibril-bound proteases. Desmin became degraded in isolated 12 h-myofibrils after the myofibrils were incubated with Ca^{2+}, and the degradation products increased with the extended incubation time (Fig. 4, Study I). In myofibrils isolated from meat with different storage time, the content of intact desmin declined after incubation of myofibrils with 0.05 mM Ca^{2+} than that of incubation without Ca^{2+}, and further decreased when the Ca^{2+} concentration was increased by a factor of 100 to 5 mM (Fig. 4, Study II). To investigate the proteolytic potential of myofibril-bound enzymes, day 6 myofibrils (with greatest amount of bound calpain-1) were incubated with increased concentration of Ca^{2+} to obtain two Ca^{2+} titration curves by the release of peptides and desmin degradation. The released peptides increased rapidly with Ca^{2+} concentration up to 0.06 mM, and thereafter it was stable in the range of 0.06-0.2 mM, followed by a

substantial increase from 0.4 to 0.8 mM. A similar change was shown by degradation of desmin. Desmin was rapidly degraded with Ca^{2+} concentration up to 0.06 mM, and subsequently more slowly to 0.4 mM, followed by a pronounced degradation to 0.6 mM. These curves showed the presence of two distinct myofibril-bound proteolytic activities corresponding to the Ca^{2+} requirements for calpain-1 and calpain-2, respectively (Fig. 5, Study II).

The effects of pre-rigor temperature of muscle (14, 22, 30 and 38 °C) and storage (1 and 4 day) on the activity of myofibril-bound calpains were assessed by degradation of desmin and troponin T, and particle size of myofibrils (Fig. 3; Tables 1 and 3, Study III). Degradation of desmin and troponin T, and myofibril fragmentation increased with the increase of temperature, storage time and Ca^{2+} concentration. On day 1, the amount of intact desmin in muscle incubated at 14 and 38 °C was similar after incubation of myofibrils without Ca^{2+}, and was significantly higher than that of 22 and 30 °C. However, in muscle at 14, 22 and 30 °C, intact desmin and particle size of myofibrils were significantly reduced after myofibrils were incubated with 0.04 mM Ca^{2+} compared to that of incubation without Ca^{2+}, and further decreased with 0.8 mM Ca^{2+}. In contrast, in meat at 38 °C, a slight decrease of intact desmin and no change in particle size of myofibrils were observed after incubation of myofibrils with 0.04 mM Ca^{2+} compared with that of incubation without Ca^{2+}, but they significantly declined with 0.8 mM Ca^{2+}. The level of intact desmin in meat at 14, 22 and 30 °C decreased on day 4 compared to that of day 1, while only a small decrease at 38 °C was observed between these two days. On day 4, the effect of increased Ca^{2+} concentration on desmin degradation was similar to that of day 1. Desmin degradation and reduction of particle size increased after incubation of myofibrils with Ca^{2+} in the order 0.8 mM > 0.04 mM > 0. Troponin T degradation products in meat at 38 °C were slightly less than that of other temperatures after incubation of myofibrils with 0 and 0.04 mM Ca^{2+}. Degraded troponin T increased ($P < 0.05$) after myofibrils were incubated with 0.8 mM Ca^{2+} compared to that of incubation with lower Ca^{2+} concentrations, and this increase became grater with increased temperatures. Compared with day 1, degradation of troponin T demonstrated a similar trend, but the degree of degradation was higher on day 4.

5.3. Effect of pre-rigor temperature on meat quality regarding proteolysis and WHC of meat

In the current thesis, purge loss of meat after pre-rigor incubation with increased temperature was measured during the period of the refrigerated storage. Purge loss of pork became greater with increased pre-rigor temperature and storage ($P < 0.01$) (Tables 1 and 2, Study III). There was no significant difference in purge loss of muscles at 22 and 30 °C, while they were significantly higher and lower than that of muscle at 14 and 38 °C, respectively. In addition, no difference ($P > 0.05$) in cook loss was observed in meat incubated at 14, 22 and 30 °C, but they were significantly higher compared to that of muscle at 38 °C. Total loss of pork was not influenced by the pre-rigor incubation temperature ($P > 0.05$). After incubation of myofibrils with different amount of Ca^{2+}, no significant difference in WHC of myofibrils from meat at 14, 22 and 30 °C was found, while they were significantly higher than that of 38 °C on day 1. For each of the incubation temperatures on day 1, a greater WHC ($P < 0.05$) of myofibrils was observed after incubation of myofibrils with 0.8 mM Ca^{2+} than that of incubation with 0 and 0.04 mM Ca^{2+}. For meat incubated at 30 and 38 °C, WHC of myofibrils on day 4 decreased compared with that of day 1 after incubation of myofibrils with 0.8 mM Ca^{2+}.

6. General discussion

6.1. Ca^{2+} and its role in the formation of myofibril-bound calpains

Proteolytic activity of calpain-2 against myofibrils

It has been generally accepted that the calpain system contributes to postmortem proteolysis and the resultant meat tenderization process during storage (Goll et al., 2003; Huff-Lonergan & Lonergan, 2005). Ca^{2+} plays an important role in the activation of calpains and its concentration influences the regulation of calpain activity. In the current thesis, the required Ca^{2+} concentration for half-maximal activity of calpain-2 against myofibrils was 290 µM (Fig. 3A, Study I). In contrast, higher Ca^{2+} concentrations of 380 µM (Barrett et al., 1991) and 1000 µM (Ceña, Jaime, Beitrán, & Roncalés, 1992) were reported to be required for half-maximal activity of calpain-2 by using myofibrils from rabbit and lamb skeletal muscle as substrate, respectively. In a study by Barrett et al. (1991), no significant difference in Ca^{2+} requirements for half-maximal hydrolysis were observed among skeletal muscle myofibrils, casein and other protein-or peptide-substrates. The reported Ca^{2+} concentration requirements for half-maximal activity are in the range of 3- 50 µM for calpain-1 and 400-800 µM for calpain-2, which is much higher compared with the level of free Ca^{2+} (5-300 nM) in living cells (Goll et al., 2003). However, the concentration of free Ca^{2+} in muscle will increase after slaughter and a concentration of 50-400 µM free Ca^{2+} has been found in muscle during postmortem storage (Geesink, Taylor, Bekhit, & Bickerstaffe, 2001; Zhang, Liu, Wang, Kang, Zhou, & Zhang, 2018; Zhang & Ertbjerg, 2018). In the current thesis, the concentration of free Ca^{2+} in pork was observed to increase with storage time and higher pre-rigor temperature (Table 2, Study III). The pre-rigor incubation of muscle at 38 °C caused a significant increase of free Ca^{2+} concentration, leading to more than 230 µM of free Ca^{2+} at 8 h postmortem in pork which was 4-5 times higher compared to that of meat at 14 °C. Hwang et al. (2004) found that the free Ca^{2+} concentration in beef incubated at 36 °C increases faster compared with that of meat at 5 and 15 °C in first 5 h incubation, and thereafter it increases slower in the rest of incubation period, while free Ca^{2+} reached around 130 µM in beef at all incubation temperatures at the end of incubation. Similarly, a faster increase of free Ca^{2+} concentration during storage has been observed in pork after freezing-then-thawing process, and a three-fold increase in Ca^{2+} concentration on day 1 caused by the freezing-thawing process of pork was observed by Zhang & Ertbjerg (2018), resulting in more than 400 µM Ca^{2+} was measured in the sarcoplasm and thereby early activation of calpain-1 and calpain-2.

Characteristics of myofibril-bound protease

In the current thesis, there was Ca^{2+}-dependent proteolytic activity within myofibrils which was able to cause proteolysis of myofibrils. This was evidenced by observing the presence of increased desmin and troponin T degradation products, smaller particle size and greater amount of peptides released from myofibrils after incubation of isolated myofibrils with Ca^{2+} than that of without Ca^{2+}. This myofibril-bound protease was activated by Ca^{2+} and its proteolytic activity was dependent on the Ca^{2+} concentration as greater activity was observed upon incubation of isolated myofibrils with increased concentrations of Ca^{2+} (Fig. 5, Study I; Figs. 4 and 5, Study II; Fig 3, Table 3, Study III). Hydrolysis of myofibrillar proteins caused by this or these myofibril-associated enzyme(s) shows the same pattern as incubations with calpains. To further identify the proteolytic potential of myofibril-bound enzymes, two Ca^{2+} titration curves were conducted as observed by desmin degradation and general release of peptides. The curves indicated activation of two distinct proteolytic activities by Ca^{2+} in the range of 30-60 μM and 400-800 μM, respectively (Fig. 5, Study II), which are similar to the reported Ca^{2+} concentration requirements for half-maximal activity of calpain-1 (3- 50 μM) and calpain-2 (400-800 μM) (Goll et al., 2003). Collectively, these results suggest that the myofibril-bound protease is calpain, and that not only calpain-1, but also calpain-2 associate to myofibrils during aging and thereafter lead to degradation of myofibrillar proteins including desmin and troponin T. Similarly, Zeng et al. (2017) reported increased desmin degradation and peptides release after isolated myofibrils were incubated with increased Ca^{2+} concentrations. In addition, even more desmin degradation and peptides released from myofibrils were observed when they incubated purified calpain-2 with isolated myofibrils and Ca^{2+} than that of isolated myofibrils were only incubated with Ca^{2+}. They thus suggested that the myofibril-bound proteases in their study were calpain-1 and calpain-2.

Effect of Ca^{2+} on the binding of calpain-2 to myofibrils and activity of myofibril-bound calpains

The free Ca^{2+} is the activator for calpains. Ca^{2+} is also necessary for calpains to bind to myofibrils and degrade myofibrillar proteins as shown in the current thesis. Extensive desmin degradation was found after a brief exposure of isolated myofibrils to purified calpain-2 and Ca^{2+} followed by an immediate isolation and incubation of the myofibrils with added Ca^{2+}, compared with that of isolated myofibrils were only incubated with Ca^{2+} (Figs. 4A and 4C, Study I). These results indicate that Ca^{2+} induces purified calpain-2 bind to the isolated myofibrils and that the myofibril-bound calpain-2 is proteolytically active and able to further degrade desmin. However, no further degradation of desmin

was observed after Ca^{2+} was substituted by EGTA in the binding process and thereafter incubation of the myofibrils with Ca^{2+}, compared with that of isolated myofibrils only incubated with Ca^{2+} (Figs. 4A and 4B, Study I). These results indicate that purified calpain-2 will not bind to isolated myofibrils in the absence of Ca^{2+}. The binding of calpain-2 to isolated myofibrils became greater with increased concentration of free Ca^{2+}, as shown by a decrease of calpain-2 activity remaining in the supernatant fraction after increased Ca^{2+} was added to induce the binding of calpain-2 to isolated myofibrils (Fig. 3B, Study I). In addition, there was more desmin degradation by the myofibril-bound calpain-2 after the binding process and incubation of the myofibrils with specified Ca^{2+} concentration (Fig. 5, Study I). The half maximal free Ca^{2+} requirement for binding of purified calpain-2 to isolated myofibrils was 0.6 mM (Fig. 3B, Study I). In accord, in the current thesis, the content of myofibril-bound calpain-1 in postmortem muscle increased at early refrigerated storage accompanied with the reported increase of free Ca^{2+} in the sarcoplasm during storage (Fig. 1, Study II). Furthermore, a faster increase of the amount of myofibril-bound calpain-1 and concentration of free Ca^{2+} were observed in muscles at higher pre-rigor temperature (Fig. 2 and Table 2, Study III). Collectively, the increased amount of myofibril-bound clapain-1 in postmortem meat was suggested to be the result of the increased free Ca^{2+} concentration in the sarcoplasm. It thus can be hypothesized that translocation of calpain-1 from the sarcoplasm to the myofibrils is initiated by a gradually increased concentration of free Ca^{2+} during postmortem storage. After calpain-1 binding to the myofibrils, myofibrillar proteins, such as desmin and troponin T, can be degraded by myofibril-bound calpain-1. Added purified calpain-2 started to bind to isolated myofibrils once Ca^{2+} was added into the suspension, and thereafter more desmin was found to be degraded by the myofibril-bound calpain-2 (Figs. 4 and 5, Study I). The binding of purified calpain-2 to isolated myofibrils and proteolysis caused by myofibril-bound calpain-2 can be observed at 50 μM and 120 μM free Ca^{2+}, respectively (Fig. 3, Study I). Combined with the reported level of Ca^{2+} concentration in meat during storage, especially in the later storage period, partial calpain-2 activation and association to myofibrils can be speculated to be initiated by the gradually increased Ca^{2+} concentration in postmortem meat.

Stability of myofibril-bound calpain-2

In order to detect if purified calpain-2 was tightly bound to the isolated myofibrils, multiple washings were applied to the myofibrils after Ca^{2+} inducing the binding procedure (Fig. 6, Study I). There was still sufficient proteolytic activity retaining in the myofibrils to degrade desmin after the myofibrils subjected to several times of washing with a large excess of washing buffer, although this activity tended to decrease. In the current assay, even after the 4-times washing, the amount of intact desmin (Fig. 6, Study I) was much less than that of isolated myofibrils which were directly incubated with Ca^{2+} for 2 h (Fig. 4A, Study I), indicating that except the active calpains that were with the myofibrils before the binding process, there was still added calpain-2 bound to the isolated myofibrils after 4 washings which was able to degrade desmin in the presence of Ca^{2+}. The observation of declined amounts of desmin degradation products shows that there will be a gradual decrease in activity of myofibril-bound calpain-2 with increasing number of washings. Nevertheless, these observations suggest that calpain-2 binds tightly to the myofibrils following activation by Ca^{2+}. On the other hand, regarding the observation of the reduced content of desmin degradation products, it is uncertain whether it was a result of decreased amount of myofibril-bound calpain-2, or whether calpain-2 was still associated to the myofibrils but partially lost its activity during the repeated washing processes. In addition, in our preliminary experiments, some calpain was still bound to the myofibrils even after 6 times washing, and after activation by sufficient concentrations of free Ca^{2+}, these myofibril-bound calpains were proteolytically active and able to degrade desmin and produce the same pattern of desmin degradation products as calpain-2 (results not shown). Similarly, calpain-1 remained bound to myofibrils even after myofibrils had been washed 14 times (Boehm et al., 1998) or 11 times (Delgado et al., 2001b), each study containing two washings with 1% Triton X-100 to remove membranous material. In most previous studies that have reported the presence of myofibril-bound calpains, the preparation process of isolated myofibrils generally included homogenization and several times washing with a large excess of buffer (Delgado et al., 2001a; Melody et al., 2004; Rowe et al., 2004b), and part of the studies even used some detergent such as 1% triton (Boehm et al., 1998; Delgado et al., 2001b) and acetone (Neath et al., 2007) before the detection of myofibril-bound calpains by methods like western blot. Thus, the presence of calpains in the myofibrillar fractions after these intense steps for extraction of myofibrils has already indicated that at least some calpains are tightly bound to myofibrils.

6.2. Hypothesis for postmortem proteolysis by myofibril-bound calpains

Changes in contents of sarcoplasmic and myofibril-bound calpain-1

In the current thesis, in order to investigate the effects of refrigerated storage, and high pre-rigor temperature incubation with subsequent refrigerated storage on translocation and proteolytic activity of calpains in porcine LT muscle, the content of calpains in the sarcoplasm and myofibrils, and activity of myofibril-bound calpains in relation to the degradation of myofibrillar proteins were determined. The content of sarcoplasmic calpain-1 decreased in meat during cold storage, while the content of myofibril-bound calpain-1 initially increased ($P < 0.05$) to 17% of 12-h sarcoplasmic calpain-1 at day 6 and thereafter decreased (Fig. 1, Study II), indicating that calpain-1 gradually translocated from the sarcoplasm to the myofibrils during the initial 6-day postmortem storage. In agreement with our results, Melody et al. (2004) and Rowe et al. (2004a) found that calpain-1 gradually translocated from the sarcoplasm to the myofibrils by observing a decreased sarcoplasmic calpain-1 content and increased myofibril-bound calpain-1 content in porcine and bovine muscle, respectively. Other studies also have reported that calpain-1 will become progressively bound to the myofibrils during postmortem storage (Boehm et al., 1998; Delgado et al., 2001b). In the current thesis, higher pre-rigor temperature incubation led to a faster decrease of the protein level of sarcoplasmic calpain-1 and a faster increase of myofibril-bound calpain-1. The amount of myofibril-bound calpain-1 was reduced on day 4 compared with that of day 1, and it reached 15%, 17% and 24% of that of 1 h-sarcoplasmic calpain-1 on day 1 in muscle incubated at 14, 22 and 30 °C, respectively (Table 2, Study III). These results indicate that the translocation of calpain-1 from the sarcoplasm to the myofibrils can be accelerated by high pre-rigor temperature of muscle.

Extractable activity and sarcoplasmic content of calpain-1

A substantial decrease in extractable activity of calpain-1 within the first one or two days postmortem has been reported earlier (Koohmaraie, Seidemann, Schollmeyer, Dutson, & Crouse, 1987; Pomponio & Ertbjerg, 2012). In the study of Delgado et al. (2001b), the extractable activity of calpain-1 in sheep *longissimus dorsi* muscle on day 3 was found to decrease to 19% of its initial activity during 4 °C storage and it was no longer detectable after 10-day postmortem storage. Camou et al. (2007) also found that in beef stored at 4 °C that calpain-1 activity decreases nearly to zero in 48 h postmortem. The high pre-rigor temperatures of muscle were reported to accelerate the decrease of extractable

activity of calpain-1 and calpain-2 (Boehm et al., 1998; Liu et al., 2014; Pomponio & Ertbjerg, 2012). By contrast, in the current thesis, the results of western blots demonstrated that the amount of calpain-1 in the sarcoplasm reduced much slower than the mentioned substantial decrease of the extractable activity of calpain-1 in meat during postmortem storage, and there was still 33% sarcoplasmic calpain-1 after 12-day cold storage (Fig. 1, Study II). Similarly, no extractable calpain-1 activity in beef could be observed by casein zymography after a 15-day storage at 4 °C, while a large amount of calpain-1 was detected in the sarcoplasm by western blot (Rowe et al., 2004a). The difference between sarcoplasmic content and extractable activity of calpain-1 could likely be attributed to that although calpain-1 loses its extractable activity, part of it still presents in the sarcoplasm and can be detected by its specific antibody. In contrast to the current thesis, where the combined native and autolyzed forms of calpain-1 were quantified, only native calpain-1 in the sarcoplasm in lamb was quantified by Ilian, Bekhit, & Bickerstaffe (2004a) during 7-day postmortem storage and the content of native calpain-1 reduced from 50% on day 1 to nearly zero on day 3. A similar result was observed in the current thesis, a significant amount of calpain-1 was native on day 1 but autolyzed thereafter (Fig. 1, Study II; Fig. 2, Study III). Rowe et al. (2004b) likewise found in beef that native calpain-1 is present only during the first 2-day postmortem storage.

Hypothesis for myofibril-bound calpains in postmortem proteolysis

A direct link between activation of calpains by Ca^{2+} and the binding of calpains to isolated myofibrils, and the proteolytic activity of myofibril-bound calpains on myofibrillar proteins degradation was discussed in Chapter 6.1. Loss of extractable activity of calpains, can thus be hypothesized to be the result of three distinct aspects *i*) binding of calpains to structural proteins within myofibrils and other subcellular organelles (such as sarcolemma, mitochondria and sarcoplasmic reticulum), *ii*) inhibition by formation of a complex with calpastatin, and *iii*) instability caused by autolysis. It has been reported by several studies that calpain-1 progressively binds to myofibrils in pork (Melody et al., 2004), beef (Boehm et al., 1998; Rowe et al., 2004b) and lamb (Delgado et al., 2001a, 2001b) during postmortem storage. In agreement with results in the current thesis, the activity of myofibril-bound calpain-1 first increased and thereafter decreased during 10 days storage in lamb (Delgado et al., 2001b). A hypothesis (Fig. 5) can be proposed that calpains gradually binds to myofibrils induced by an increase of free Ca^{2+} in the sarcoplasm during postmortem storage followed by degradation of myofibrillar

proteins by myofibril-bound calpains, which thereby leads to a tenderization of meat. Calpains will be released from the myofibrils to the sarcoplasm once myofibril-bound calpains complete the proteolysis of available substrates in the vicinity of where they are associated in the myofibrillar proteins. It is in accordance with the results of the decreasing trend for the content of myofibril-bound calpain-1 in later postmortem storage (Fig. 1, Study II; Fig. 2, Study III), and slower reduction of sarcoplasmic calpain-1 and calpain-2 contents in later storage than that of earlier storage (Figs. 1 and 2, Study II). The involvement of calpain-1 in meat tenderization has been questioned due to that there is a time difference between the substantial reduction of extractable calpain-1 activity at early storage and proteolytic degradation of myofibrillar proteins later. Once calpain-1 binds to myofibrils, however, its extractable activity cannot be determined by in vitro assays such as standard casein assay or casein zymography, and this is reflected as a loss of extractable calpain activity. Calpain-1 will, however, be proteolytically active in the form of myofibril-bound calpain-1 as shown by current thesis. Therefore, activity of calpain-1 and calpain-2 and their contribution to meat tenderization during postmortem storage will be underestimated if based only on the changes in extractable calpain activity. That may be the reason why whole muscle extracts were used in some studies to evaluate proteolytic activity of calpains (Kim et al., 2010; Ma & Kim, 2020). The activity of myofibril-bound calpain-1 has also been measured in some studies, while the activity has been relatively low considering the large amount of calpain-1 that will be bound to myofibrils during postmortem storage (Boehm et al., 1998; Delgado et al., 2001b). This is likely because they used hydrolysis of casein (labeled with FITC or ^{14}C) as the indicator to evaluate the activity for myofibril-bound calpain-1. It seems that myofibril-bound calpains cannot effectively hydrolyze exogenous proteins, which may be attributed to steric hindrance for access to the active sites in myofibril-associated calpains. Additionally, it has been reported by Boehm et al. (1998) and Delgado et al. (2001b) that proteolytic activity of myofibril-bound calpain-1 cannot be inhibited by calpastatin. Once calpains bind to myofibrils, the activity will thus not be influenced by calpastatin and this could be the reason why structural proteins are degraded by calpain-1 at early storage even when a large amount of calpastatin is present. The influence of calpastatin on meat tenderness may be attribute to its effect on the amount of calpains becoming associated to myofibrils.

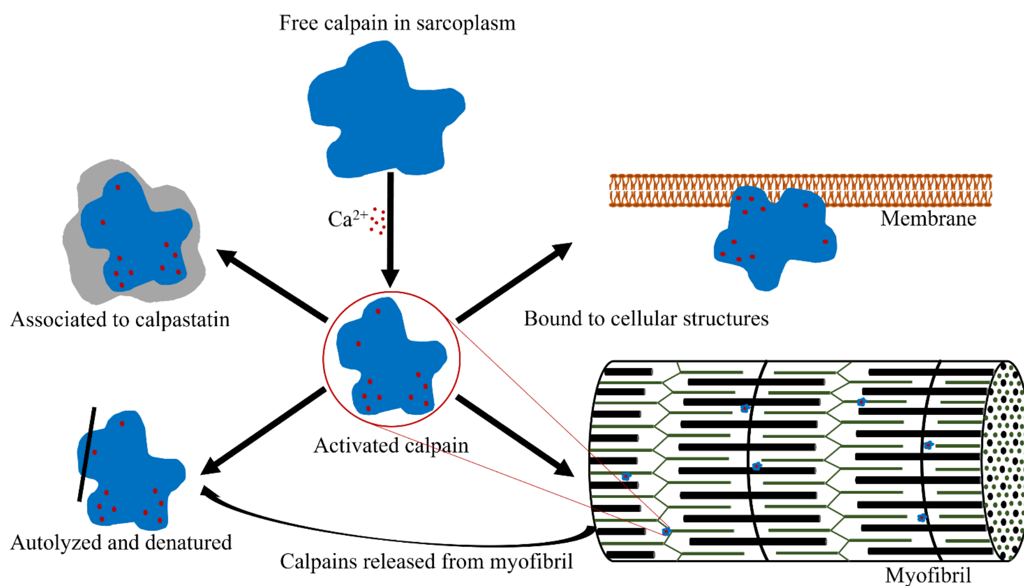

Fig. 5. Hypothesis illustrates the process of calpain activation, binding to myofibrils, degradation of structural proteins by the myofibril-bound calpain and then released from myofibrils back to the sarcoplasm. In postmortem muscle, calpains were activated by an increased Ca^{2+} concentration in the sarcoplasm. Next, the activated calpains bound to membranes, mitochondria or bound to myofibrils; the latter process led to degradation of structural proteins; finally calpains released from myofibrils to the sarcoplasm after the proteolytic degradation. Part of the activated calpains can also bind to calpastatin or lose activity following autolysis.

It has been widely accepted that calpain-1 is activated during early postmortem storage and has an important role in tenderization of meat, while calpain-2 activity is relatively stable during storage (Koohmaraie et al., 1987; Veiseth et al., 2001). However, reduction of calpain-2 activity during storage has been observed in several studies (Boehm et al., 1998; Camou et al., 2007; Phelps et al., 2016; Zhang & Ertbjerg, 2018). In the current thesis, a decrease for calpain-2 content in the sarcoplasm in pork during storage was found to be similar to that of calpain-1, although its decrease was relatively slower, and the difference between the decreasing trend of calpain-1 and calpain-2 diminished gradually during later postmortem storage (Figs. 1 and 2, Study II). In addition, the degree of proteolysis was higher when isolated myofibrils were incubated with 800 μM Ca^{2+} than that of 40 μM Ca^{2+} by the observation of more degradation of desmin and troponin T and smaller particle size, indicating that calpain-2 was also bound to myofibrils in meat and the myofibril-bound calpain-2 was proteolytically active to degrade myofibrillar proteins (Fig. 3, Table 3, Study III). Similar results were observed in study II (Fig. 4) and in a study by Zeng et al. (2017) that more desmin degradation products were found when incubation of isolated myofibrils with higher Ca^{2+} concentrations. Conclusively, by

combining the results of the binding of calpain-2 to isolated myofibrils and its Ca^{2+} requirement (Figs. 3B, 4 and 5, Study I), the Ca^{2+} titration curve (Fig. 5, Study II) and free Ca^{2+} concentration in the current thesis (Table 2, Study III), we suggest that calpain-2 undergoes a similar process as calpain-1 if the free Ca^{2+} reach a sufficient high level.

6.3. Effects of myofibril-bound calpains induced proteolysis and protein denaturation on meat quality

Protein denaturation, WHC and shear force

The capability of fresh meat to retain its own water is defined as its WHC (Pearce et al., 2011). Purge loss (drip loss) has often been used to reflect the WHC of postmortem meat (Savage, Warriss, & Jolley, 1990). In the current thesis, purge loss was found to increase with refrigerated storage and increased pre-rigor temperature (14-38 °C) (Table 2, Study III). In accord, the poorest WHC of myofibrils was observed on day 1 and day 4 in muscles at 38 °C. Similarly, several studies have documented that WHC of meat decreased with the increased pre-rigor temperature by observing greater purge losses, and this was attributed to the reduced sarcomere volume and increased extent of protein denaturation caused by the high pre-rigor temperature with fast pH decline (Geesink et al., 2000; Liu et al., 2014; Warner, Kerr, Kim, & Geesink, 2014; Zhu et al., 2013; Zhu, Ruusunen, Gusella, Zhou, & Puolanne, 2011). However, Mungure, Bekhit, Birch, & Stewart (2016) and Devine, Wahlgren, & Tornberg (1999) have reported that rigor temperature did not influence WHC of beef during storage. In the current thesis, no evident change of WHC of myofibrils between day 1 and day 4 was found ($P > 0.05$), although purge loss of muscle significantly increased (Table 3, Study III). By contrast, Zhang & Ertbjerg (2018) found that water-holding of pork decreases during chilled storage, as observed by the reduced WHC of myofibrils and increased purge loss. Additionally, the decrease in water-holding was significantly exacerbated by the freezing-thawing process in the study of Zhang & Ertbjerg (2018), and they partly attributed it to the protein denaturation induced by the freezing-thawing process. As reviewed by Pearce et al. (2011), the majority of water loss within meat during postmortem storage is because the mobility of water to the extracellular space driven by factors such as decreased myofibrillar volume, and a continual release of protein-bound water induced by ongoing denaturation of proteins. Myosin is very susceptible to temperature and it denatures substantially during the pre-rigor period, especially with the combination of high temperature and fast pH decline (Penny, 1967). Also,

denaturation of myosin occurs during storage due to its exposure to the extreme conditions (low pHs) in post-rigor muscle (Offer, 1991). Denaturation of myosin heads leads to decreased WHC of muscle by a significant contribution to the lateral shrinkage of myofibrils and a reduced ability of denatured myosin to associate water (Offer & Knight, 1988). In the current study, the results of no decrease in WHC of myofibrils from day 1 to day 4 but increased purge loss in the current thesis, could be due to that the higher pre-rigor temperature combined with fast pH drop induce much more early protein denaturation and other changes that reduces WHC of myofibrils in the pre-rigor phase than in muscle with lower pre-rigor temperature, and thus most of the decrease in WHC of myofibrils occurred early postmortem. The increased purge loss in pork at 38 °C early postmortem may be due to substantially reduced WHC of myofibrils and thus increased extracellular water, while during later storage it is possibly caused by extracellular water running out but not the further decrease of the WHC of myofibrils. In accord, the initial purge loss of muscles at 38 °C was 8% on day 1 which was significantly higher than that of muscle at lower temperatures with around 1% - 2% purge loss, and it rapidly increased on day 2 while gradually increased until day 4. However, the purge loss in muscle at lower temperatures increased linearly during whole 4-day storage (Table 2, Study III). The low WHC of muscles at 38 °C may also be attribute to the aspect that high pre-rigor temperature induced protein denaturation limits the calpain activity and postmortem proteolysis.

In the current study, after 4-day storage, the lowest shear force value was observed in muscles pre-rigor incubated at 38 °C. However, it seems that the softness of samples incubated at 38 °C was not due to postmortem proteolysis since there were only limited activation of calpain-1 and less protein degradation than that of at the lower incubation temperatures. Liu et al. (2014) also reported that pork samples pre-rigor incubated at 40 °C had the lowest shear force value after 3-day storage compared with that of samples incubated at 0, 10, 20 and 30 °C. They attributed this decreased toughness of muscle incubated at 40 °C to the proteolysis caused by earlier activation of calpain-1 and calpain-2 by the observation of earlier disappearance of native calpains on the casein zymography gel. By contrast, in Study III, there were less activation of calpain-1 as indicated by western blot analysis and less degradation of desmin and troponin T. These observations suggest that earlier activation of calpains and proteolysis are not the reasons for the lower shear force of samples incubated at 38 °C. The earlier disappearance of calpains could be due to the precipitation of calpains onto myofibrils (Study III, Fig. 2). Liu et al. (2014) also measured sarcomere length and found that muscles incubated at 0 and 40 °C had the lowest sarcomere length, and they thereby suggested that heat shortening occurred to the

muscles pre-rigor incubated at 40 °C. Ertbjerg and Puolanne (2017) reviewed that fracture of super-contracted sarcomeres could very well accounted for the reduced toughness at muscle shortening > 40%. Taken together, it can thus be speculated that the softness of muscles pre-rigor incubated at 38 °C could be due to super-contraction induced by heat shortening.

Myofibril-bound calpains caused proteolysis and changes in WHC

As reviewed by Kim et al. (2014), except the rate and extent of pH decline, the denaturation of proteins and sarcomere shortening, the role of proteolysis in WHC of postmortem meat has also been well documented. In the current thesis, after myofibrils were incubated with Ca^{2+}, WHC of myofibrils increased in meat at all rigor temperatures (Table 3, Study III). At the same time, increased proteolysis was found as observed by the more degradation of desmin and troponin T and smaller particle size after this incubation (Fig. 3; Table 3, Study III). These results suggest that there are calpains bound to myofibrils which can cause degradation of myofibrillar proteins in the presence of sufficient Ca^{2+}, which in turn may affect the WHC of myofibrils. Similarly, Zeng et al. (2017) found an increased WHC of myofibrils after the incubation of myofibrils with Ca^{2+}, and they attributed it to proteolysis induced swelling of myofibrils by the observation that peptides release and desmin degradation increased. In addition, WHC of myofibrils and proteolysis further increased when they added purified calpain-2 into the incubation system. This shows that the myofibril-bound calpains have the similar proteolytic activity in postmortem muscle compared to that of the purified calpains. Melody et al. (2004) also reported increased WHC following myofibril-bound calpain-1 induced proteolysis as observed by earlier appearance and more abundant myofibril-bound calpain-1 accompanied with higher degree of degradation of desmin, titin and nebulin and lower drip loss in *psoas major* in beef. It has been well recognized that the proteolytic potential has a relevant role in increasing the WHC of muscle during aging. The calpain system has been generally accepted as an important contributor to proteolysis and thereby meat tenderization (Bee et al., 2007; Huff-Lonergan & Lonergan, 2005; Koohmaraie & Geesink, 2006; Kristensen & Purslow, 2001). The effects of proteolysis on improved WHC of meat during postmortem aging can be hypothesized to three aspects: *i*) swelling of muscle fibers and thereafter water flow inside muscle fibers (Huff-Lonergan & Lonergan, 2005; Kristensen & Purslow, 2001), *ii*) swelling of myofibrils that subsequently lead to water flow into myofibrils (Zeng et al., 2017), and *iii*) disruption of drip channels and physically entrapping water within meat (Farouk,

Mustafa, Wu, & Krsinic, 2012). Increased WHC of meat caused by swelling of muscle fibers is likely attributable to the proteolytic disruption of the cytoskeletal connections between myofibrils, and connections between myofibrils and sarcolemma, and thereby releasing the force that induces water flow outside of cells, subsequently lead to a flow of the extracellular water to intracellular space (Bee et al., 2007; Kristensen & Purslow, 2001). Similarly, Zeng et al. (2017) hypothesized that desmin degradation and α-actinin release which occur in and around Z-disk remove restrictions on myofibrils and result in the swelling of myofibrils followed by water flowing from the space outside myofibrils to inside of myofibrils.

7. Conclusions

In vitro, Ca^{2+} induces purified calpain-2 to bind to isolated myofibrils and the association between calpain-2 and myofibrils is relatively tight. After binding of calpain-2 to isolated myofibrils, the myofibril-bound calpain-2 remains proteolytically active to cause desmin degradation in the presence of Ca^{2+}. The binding of calpain-2 to isolated myofibrils and purified-calpain-2 proteolysis against myofibrils increase with greater concentration of free Ca^{2+}, and the half-maximal Ca^{2+} requirements for them are 0.60 mM and 0.29 mM, respectively.

The sarcoplasmic content of calpain-1 and calpain-2 in pork LT muscle decreases with refrigerated storage, while the amount of myofibril-bound calpain-1 becomes greater until day 6 cold storage, suggesting that calpain-1 translocates from sarcoplasm to myofibrils during early postmortem storage, and translocation of calpain-2 is probable following the same pattern as that of calpain-1 but occurs later postmortem. High muscle temperature before rigor leads to a faster translocation of calpains from sarcoplasm to myofibrils, in parallel with an increase of free Ca^{2+} concentration in muscle, suggesting that the binding of calpains to myofibrils within meat is similar to that of the *in vitro* assay and it increases with higher level of Ca^{2+} in the sarcoplasm during aging.

Proteolysis of myofibrillar proteins by myofibril-bound calpains increases upon incubation of isolated myofibrils with greater Ca^{2+} concentration as evidenced by higher degree of desmin and troponin T degradation, more released peptides and higher myofibril fragmentation. Ca^{2+} titration curves show that myofibrils have two distinct myofibril-bound proteolytic activities corresponding to the reported requirements of Ca^{2+} for calpain-1 and calpain-2, respectively. Collectively, these results demonstrate that both calpain-1 and calpain-2 become bound to myofibrils during postmortem storage and that the myofibril-bound calpains are proteolytically active and able to degrade myofibrillar proteins such as desmin and troponin T. A hypothesis is proposed principally based on the involvement of myofibril-bound calpains in postmortem proteolysis and the resultant meat tenderization.

8. Future perspectives

The thesis showed that the binding of calpains to isolated myofibrils increased with an increase of added Ca^{2+} and also the amount of myofibril-bound calpains within pork LT muscle became greater with increased concentration of Ca^{2+} in the sarcoplasm during chilled storage. To better demonstrate the relationship between the level of Ca^{2+} and the content of myofibril-bound calpains in meat, pre-rigor incubation with increased temperature was applied to increase the concentration of free Ca^{2+} in pork at early storage when there were large amounts of native calpains. However, the goal was not fully achieved due to the possible presence of a relative large amount of denatured proteins and limited activity of calpain-1 under condition of high pre-rigor temperature with fast pH decline. Thus, other meat models that can increase the level of sarcoplasmic Ca^{2+} during early postmortem storage, such as the freezing-then-thawing process, ultrasound or electrical stimulation should be considered.

Translocation of calpain-1 from sarcoplasm to myofibrils during early postmortem storage of pork was shown to occur in the current thesis as evidenced by the amount of calpain-1 decline in the sarcoplasm and increased amount becoming bound to myofibrils, which was shown by western blot followed by quantification of the protein bands. However, this method was not available for the detection of myofibril-bound calpin-2 which possibly was due to the low sensitivity of the antibody used against calpain-2. Hence, a more sensitive antibody or other methods, for instance immunofluorescence microscopy should be developed to better quantify calpains in different factions.

The proteolytic activity of myofibril-bound calpains was illustrated by the degree of myofibrillar proteins degradation upon incubation of the isolated myofibrils with added Ca^{2+}. However, it is not an efficient method to reflect the proteolytic potential of myofibril-bound calpains. Additionally, greater amounts of calpain-1 becoming bound to myofibrils was observed in PSE-like pork. However, a large part of the calpain-1 is speculated to have precipitated onto the myofibrils and it is difficult to distinguish the proportions of the denatured calpains and the proteolytically active calpains in the myofibril fraction. Therefore, a more direct and accurate measurement to reveal the proteolytic activity of myofibril-bound calpains should be designed in the future, and then establish the correlation between activity of myofibril-bound calpains and the texture of PSE meat.

References

Ariyoshi, H., Shiba, E., Sakon, M., Kambayashi, J., Yoshida, K., Kawashima, S., & Mori, T. (1993). Translocation of human platelet calpain-I. *Biochemistry and molecular biology international, 30*(1), 63-72.

Badugu, R., Garcia, M., Bondada, V., Joshi, A., & Geddes, J. W. (2008). N terminus of calpain 1 is a mitochondrial targeting sequence. *Journal of Biological Chemistry, 283*(6), 3409-3417.

Baek, K. H., Yu, H. V., Kim, E., Na, Y., & Kwon, Y. (2016). Calcium influx-mediated translocation of m-calpain induces Ku80 cleavage and enhances the Ku80-related DNA repair pathway. *Oncotarget*, *7*(21), 30831-30844.

Bao, Y., & Ertbjerg, P. (2019). Effects of protein oxidation on the texture and water-holding of meat: a review. *Critical Reviews in Food Science and Nutrition*, *59*(22), 3564-3578.

Barido, F. H., & Lee, S. K. (2021). Changes in proteolytic enzyme activities, tenderness-related traits, and quality properties of spent hen meat affected by adenosine 5′-monophosphate during cold storage. *Poultry Science*, *100*(5), 101056.

Baron, C. P., Jacobsen, S., & Purslow, P. P. (2004). Cleavage of desmin by cysteine proteases: Calpains and cathepsin B. *Meat Science, 68*(3), 447-456.

Barrett, M. J., Goll, D. E., & Thompson, V. F. (1991). Effect of substrate on Ca^{2+}-concentration required for activity of the Ca^{2+}-dependent proteinases, μ- and m-calpain. *Life Sciences, 48*(17), 1659-1669.

Bee, G., Anderson, A. L., Lonergan, S. M., & Huff-Lonergan, E. (2007). Rate and extent of pH decline affect proteolysis of cytoskeletal proteins and water-holding capacity in pork. *Meat Science, 76*(2), 359-365.

Bendall, J. R., & Wismer-Pedersen, J. (1962). Some properties of the fibrillar proteins of normal and watery pork muscle. *Journal of Food Science, 27*(2), 144-159.

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E.-D. A. (2018). Role of calpain system in meat tenderness: A review. *Food Science and Human Wellness, 7*(3), 196-204.

Blanchard, H., Grochulski, P., Li, Y., Arthur, J. S. C., Davies, P. L., Elce, J. S., & Cygler, M. (1997). Structure of a calpain Ca^{2+}-binding domain reveals a novel EF-hand and Ca^{2+}-induced conformational changes. *Nature Structural Biology, 4*(7), 532-538.

Bloch, R. J., Capetanaki, Y., O'Neill, A., Reed, P., Williams, M. W., Resneck, W. G., . . . Ursitti, J. A. (2002). Costameres: Repeating Structures at the Sarcolemma of Skeletal Muscle. *Clinical Orthopaedics and Related Research*, *403*, S203-S210.

Boehm, M. L., Kendall, T. L., Thompson, V. F., & Goll, D. E. (1998). Changes in the calpains and calpastatin during postmortem storage of bovine muscle. *Journal of Animal Science, 76*(9), 2415-2434.

Calkins, C. R., & Seideman, S. C. (1988). Relationships among calcium-dependent protease, cathepsins B and H, meat tenderness and the response of muscle to aging. *Journal of Animal Science, 66*(5), 1186-1193.

Camou, J. P., Marchello, J. A., Thompson, V. F., Mares, S. W., & Goll, D. E. (2007). Effect of postmortem storage on activity of μ- and m-calpain in five bovine muscles. *Journal of Animal Science, 85*(10), 2670-2681.

Capetanaki, Y., Milner, D. J., & Weitzer, G. (1997). Desmin in muscle formation and maintenance: knockouts and consequences. *Cell Structure and Function, 22*(1), 103-116.

Ceña, P., Jaime, I., Beitrán, J. A., & Roncalés, P. (1992). Proteolytic activity of isolated lamb calpains on myofibrils under the conditions of pH, Ca^{2+} concentration and temperature existing in postmortem muscle. *Zeitschrift für Lebensmittel-Untersuchung und Forschung, 194*(3), 248-251.

Chang, H., Sheng, J.-J., Zhang, L., Yue, Z.-J., Jiao, B., Li, J.-S., & Yu, Z.-B. (2015). ROS-induced nuclear translocation of calpain-2 facilitates cardiomyocyte apoptosis in tail-suspended rats. *Journal of Cellular Biochemistry*, *116*(10), 2258-2269.

Chelko, S. P., Keceli, G., Carpi, A., Doti, N., Agrimi, J., Asimaki, A., . . . Paolocci, N. (2021). Exercise triggers CAPN1-mediated AIF truncation, inducing myocyte cell death in arrhythmogenic cardiomyopathy. *Science Translational Medicine*, *13*(581), eabf0891.

Chen, L., Feng, X., Zhang, Y., Liu, X., Zhang, W., Li, C., Ullah, N., Xu, X. & Zhou, G. (2015a). Effects of ultrasonic processing on caspase-3, calpain expression and myofibrillar structure of chicken during post-mortem ageing. *Food Chemistry, 177*, 280-287.

Chen, L., Feng, X., Lu, F., Xu, X., Zhou, G, Li, Q., & Guo, X. (2011). Effects of camptothecin, etoposide and Ca^{2+} on caspase-3 activity and myofibrillar disruption of chicken during postmortem ageing. *Meat Science, 87*(3), 165-174.

Chen, L., Feng, X., Zhang, W., Xu, X., & Zhou, H. (2012). Effects of inhibitors on the synergistic interaction between calpain and caspase-3 during post-mortem aging of chicken meat. *Journal of Agricultural and Food Chemistry, 60*(34), 8465-8472.

Chen, L., Zhou, G., & Zhang, W. (2015b). Effects of high oxygen packaging on tenderness and water holding capacity of pork through protein oxidation. *Food and Bioprocess Technology, 8*(11), 2287-2297.

Chen, Q., Huang, J., Huang, F., Huang, M., & Zhou, G. (2014). Influence of oxidation on the susceptibility of purified desmin to degradation by μ-calpain, caspase-3 and -6. *Food Chemistry*, *150*, 220-226.

Chua, B. T., Guo, K., & Li, P. (2000). Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *Journal of Biological Chemistry, 275*(7), 5131-5135.

Claeys, E., De Smet, S., Demeyer, D., Geers, R., & Buys, N. (2001). Effect of rate of pH decline on muscle enzyme activities in two pig lines. *Meat Science, 57*(3), 257-263.

Colle, M. J., & Doumit, M. E. (2017). Effect of extended aging on calpain-1 and -2 activity in beef longissimus lumborum and semimembranosus muscles. *Meat Science, 131*, 142-145.

Colle, M. J., Nasados, J. A., Rogers, J. M., Kerby, D. M., Colle, M. M., Van Buren, J. B., Richard, R. P., Murdoch, G. K., Williams, C. J. & Doumit, M. E. (2018). Strategies to improve beef tenderness by activating calpain-2 earlier postmortem. *Meat Science, 135*, 36-41.

Cong, J., Goll, D. E., Peterson, A. M., & Kapprell, H. P. (1989). The role of autolysis in activity of the Ca2+-dependent proteinases (μ-calpain and m-calpain). *Journal of Biological Chemistry*, *264*(17), 10096-10103.

Cornforth, D. P., Pearson, A. M., & Merkel, R. A. (1980). Relationship of mitochondria and sarcoplasmic reticulum to cold shortening. *Meat Science, 4*(2), 103-121.

Dang, D. S., Buhler, J. F., Davis, H. T., Thornton, K. J., Scheffler, T. L., & Matarneh, S. K. (2020). Inhibition of mitochondrial calcium uniporter enhances postmortem proteolysis and tenderness in beef cattle. *Meat Science*, *162*, 108039.

Dang, D. S., Stafford, C. D., Taylor, M. J., Buhler, J. F., Thornton, K. J., & Matarneh, S. K. (2022). Ultrasonication of beef improves calpain-1 autolysis and caspase-3 activity by elevating cytosolic calcium and inducing mitochondrial dysfunction. *Meat Science*, *183*, 108646.

Dayton, W. R., Goll, D. E., Zeece, M. G., Robson, R. M., & Reville, W. J. (1976). A Ca2+ion-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochemistry, 15*(10), 2150-2158.

de Moura Souza, G., Coutinho, M. A. d. S., Ramos, P. M., de Oliveira, G. M., Lonergan, S. M., & Delgado, E. F. (2019). Tough aged meat presents greater expression of calpastatin, which presents postmortem protein profile and tenderization related to Nellore steer temperament. *Meat Science*, *156*, 131-138.

de Oliveira, L. G., Delgado, E. F., Steadham, E. M., Huff-Lonergan, E., & Lonergan, S. M. (2019). Association of calpain and calpastatin activity to postmortem myofibrillar protein degradation and sarcoplasmic proteome changes in bovine *Longissimus lumborum* and *Triceps brachii*. *Meat Science, 155*, 50-60.

Delgado, E. F., Geesink, G. H., Marchello, J. A., Goll, D. E., & Koohmaraie, M. (2001a). The calpain system in three muscles of normal and callipyge sheep. *Journal of Animal Science, 79*(2), 398-412.

Delgado, E. F., Geesink, G. H., Marchello, J. A., Goll, D. E., & Koohmaraie, M. (2001b). Properties of myofibril-bound calpain activity in longissimus muscle of callipyge and normal sheep. *Journal of Animal Science, 79*(8), 2097-2107.

DeMartino, G. N., Huff, C. A., & Croall, D. E. (1986). Autoproteolysis of the small subunit of calcium-dependent protease II activates and regulates protease activity. *Journal of Biological Chemistry, 261*(26), 12047-12052.

Devine, C. E., Wahlgren, N. M., & Tornberg, E. (1999). Effect of rigor temperature on muscle shortening and tenderisation of restrained and unrestrained beef *m. longissimus thoracicus et lumborum*. *Meat Science, 51*(1), 61-72.

Ding, Z., Wei, Q., Zhang, C., Zhang, H., & Huang, F. (2021). Influence of oxidation on heat shock protein 27 translocation, caspase-3 and calpain activities and myofibrils degradation in postmortem beef muscles. *Food Chemistry*, *340*, 127914.

Doumit, M. E., & Koohmaraie, M. (1999). Immunoblot analysis of calpastatin degradation: evidence for cleavage by calpain in postmortem muscle2. *Journal of Animal Science*, *77*(6), 1467-1473.

Dransfeld, E. (1994). Modelling post-mortem tenderisation—V: Inactivation of calpains. *Meat Science, 37*(3), 391-409.

Dransfield, E., Etherington, D. J., & Taylor, M. A. J. (1992). Modelling post-mortem tenderisation—II: Enzyme changes during storage of electrically stimulated and non-stimulated beef. *Meat Science, 31*(1), 75-84.

Edmunds, T., Nagainis, P. A., Sathe, S. K., Thompson, V. F., & Goll, D. E. (1991). Comparison of the autolyzed and unautolyzed forms of μ- and m-calpain from bovine skeletal muscle. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, *1077*(2), 197-208.

Elce, J. S., Hegadorn, C., & Arthur, J. S. C. (1997). Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. *Journal of Biological Chemistry*, *272*(17), 11268-11275.

Ertbjerg, P., & Puolanne, E. (2017). Muscle structure, sarcomere length and influences on meat quality: A review. *Meat Science*, *132*, 139-152.

Farouk, M. M., Mustafa, N. M., Wu, G., & Krsinic, G. (2012). The "sponge effect" hypothesis: An alternative explanation of the improvement in the waterholding capacity of meat with ageing. *Meat Science, 90*(3), 670-677.

Foster, M. W., Hess, D. T., & Stamler, J. S. (2009). Protein S-nitrosylation in health and disease: a current perspective. *Trends in Molecular Medicine*, *15*(9), 391-404.

Fritz, J. D., & Greaser, M. L. (1991). Changes in titin and nebulin in postmortem bovine muscle revealed by gel electrophoresis, western blotting and immunofluorescence microscopy. *Journal of Food Science, 56*(3), 607-610.

Fu, Q.-q., Ge, Q.-f., Liu, R., Wang, H.-o., Zhou, G.-h., & Zhang, W.-g. (2017). Influence of modified atmosphere packaging on protein oxidation, calpain activation and desmin degradation of beef muscles. *Journal of the Science of Food and Agriculture*, *97*(13), 4508-4514.

Fu, Q.-q., Liu, R., Zhang, W., Ben, A., & Wang, R. (2020). In Vitro Susceptibility of Oxidized Myosin by μ-Calpain or Caspase-3 and the Determination of the Oxidation Sites of Myosin Heavy Chains. *Journal of Agricultural and Food Chemistry*, *68*(32), 8629-8636.

Garcia, M., Bondada, V., & Geddes, J. W. (2005). Mitochondrial localization of μ-calpain. *Biochemical and Biophysical Research Communications, 338*(2), 1241-1247.

Geesink, G. H., Bekhit, A.-D., & Bickerstaffe, R. (2000). Rigor temperature and meat quality characteristics of lamb longissimus muscle. *Journal of Animal Science, 78*(11), 2842-2848.

Geesink, G. H., & Koohmaraie, M. (1999a). Effect of calpastatin on degradation of myofibrillar proteins by μ-calpain under postmortem conditions. *Journal of Animal Science, 77*(10), 2685-2692.

Geesink, G. H., & Koohmaraie, M. (1999b). Effect of calpastatin on degradation of myofibrillar proteins by μ-calpain under postmortem conditions2. *Journal of Animal Science*, *77*(10), 2685-2692.

Geesink, G. H., Kuchay, S., Chishti, A. H., & Koohmaraie, M. (2006). μ-Calpain is essential for postmortem proteolysis of muscle proteins. *Journal of Animal Science*, *84*(10), 2834-2840.

Geesink, G. H., Taylor, R. G., Bekhit, A. E. D., & Bickerstaffe, R. (2001). Evidence against the non-enzymatic calcium theory of tenderization. *Meat Science, 59*(4), 417-422.

Gil-Parrado, S., Popp, O., Knoch, T. A., Zahler, S., Bestvater, F., Felgenträger, M., Holloschi, A., Fernández-Montalván, A., Auerswald, E. A., Fritz, H., Fuentes-Prior, P., Machleidt, M. & Spiess, E. (2003). Subcellular localization and in *vivo* subunit interactions of ubiquitous μ-calpain. *Journal of Biological Chemistry, 278*(18), 16336-16346.

Glaser, T., Schwarz-Benmeir, N., Barnoy, S., Barak, S., Eshhar, Z., & Kosower, N. S. (1994). Calpain (Ca (2+)-dependent thiol protease) in erythrocytes of young and old individuals. *Proceedings of the National Academy of Sciences, 91*(17), 7879-7883.

Goll, D. E., Otsuka, Y., Nagainis, P. A., Shannon, J. D., Sathe, S. K., & Muguruma, M. (1983). Role of muscle proteinases in maintenance of muscle integrity and mass. *Journal of Food Biochemistry, 7*(3), 137-177.

Goll, D. E., Thompson, V. F., Li, H., Wei, W. E. I., & Cong, J. (2003). The calpain system. *Physiological Reviews, 83*(3), 731-801.

Gordon, A. M., Homsher, E., & Regnier, M. (2000). Regulation of contraction in striated muscle. *Physiological Reviews, 80*(2), 853-924.

Gu, X., Whipple-VanPatter, G., O'Dwyer, M., & Zeece, M. (2001). Capillary electrophoretic analysis of μ- and m-calpain using fluorescently labeled casein substrates. *Electrophoresis, 22*(11), 2336-2342.

Hanna, R. A., Campbell, R. L., & Davies, P. L. (2008). Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature*, *456*(7220), 409-412.

Hanna, R. A., Garcia-Diaz, B. E., & Davies, P. L. (2007). Calpastatin simultaneously binds four calpains with different kinetic constants. *FEBS Letters*, *581*(16), 2894-2898.

Hernando, V., Inserte, J., Sartório, C. L., Parra, V. M., Poncelas-Nozal, M., & Garcia-Dorado, D. (2010). Calpain translocation and activation as pharmacological targets during myocardial ischemia/reperfusion. *Journal of Molecular and Cellular Cardiology, 49*(2), 271-279.

Hood, J. L., Brooks, W. H., & Roszman, T. L. (2004). Differential compartmentalization of the calpain/calpastatin network with the endoplasmic reticulum and Golgi apparatus. *Journal of Biological Chemistry, 279*(41), 43126-43135.

Hood, J. L., Logan, B. B., Sinai, A. P., Brooks, W. H., & Roszman, T. L. (2003). Association of the calpain/calpastatin network with subcellular organelles. *Biochemical and Biophysical Research Communications, 310*(4), 1200-1212.

Hopkins, D. L., & Thompson, J. M. (2001). Inhibition of protease activity. Part 1. The effect on tenderness and indicators of proteolysis in ovine muscle. *Meat Science*, *59*(2), 175-185.

Horikawa, Y., Oda, N., Cox, N. J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T. H., Mashima, H., Schwarz, P. E. H., del Bosque-Plata, L., Horikawa, Y., Oda, Y., Yoshiuchi, I., Colilla, S., Polonsky, K. S., Wei, S., Concannon, P., Iwasaki, N., Schulze, J., Baier, L. J., Bogardus, C., Groop, L., Boerwinkle, E., Hanis, C. L. & Bell, G. I. (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nature Genetics, 26*(2), 163-175.

Hou, Q., Zhang, C.-y., Zhang, W.-g., Liu, R., Tang, H., & Zhou, G.-h. (2020). Role of protein S-nitrosylation in regulating beef tenderness. *Food Chemistry*, *306*, 125616.

Houbak, M. B., Ertbjerg, P., & Therkildsen, M. (2008). In vitro study to evaluate the degradation of bovine muscle proteins post-mortem by proteasome and μ-calpain. *Meat Science, 79*(1), 77-85.

Huang, F., Ding, Z., Zhang, C., Hu, H., Zhang, L., & Zhang, H. (2018). Effects of calcium and zinc ions injection on caspase-3 activation and tenderness in post-mortem beef skeletal muscles. *International Journal of Food Science & Technology*, *53*(3), 582-589.

Huang, F., Huang, M., Zhang, H., Guo, B., Zhang, D., & Zhou, G. (2014). Cleavage of the calpain inhibitor, calpastatin, during postmortem ageing of beef skeletal muscle. *Food Chemistry, 148*, 1-6.

Huang, J., & Forsberg, N. E. (1998). Role of calpain in skeletal-muscle protein degradation. *Proceedings of the National Academy of Sciences*, *95*(21), 12100-12105.

Huff-Lonergan, E., & Lonergan, S. M. (2005). Mechanisms of water-holding

capacity of meat: The role of postmortem biochemical and structural changes. *Meat Science, 71*(1), 194-204.

Huff-Lonergan, E., Mitsuhashi, T., Beekman, D. D., Parrish, F. C., Jr., Olson, D. G., & Robson, R. M. (1996). Proteolysis of specific muscle structural proteins by μ-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. *Journal of Animal Science, 74*(5), 993-1008.

Huff Lonergan, E., Zhang, W., & Lonergan, S. M. (2010). Biochemistry of postmortem muscle — Lessons on mechanisms of meat tenderization. *Meat Science, 86*(1), 184-195.

Hughes, J. M., Oiseth, S. K., Purslow, P. P., & Warner, R. D. (2014). A structural approach to understanding the interactions between colour, water-holding capacity and tenderness. *Meat Science*, *98*(3), 520-532.

Hwang, I. H., Park, B. Y., Cho, S. H., & Lee, J. M. (2004). Effects of muscle shortening and proteolysis on Warner–Bratzler shear force in beef *longissimus* and *semitendinosus*. *Meat Science, 68*(3), 497-505.

Hwang, I. H., & Thompson, J. M. (2001). The interaction between pH and temperature decline early postmortem on the calpain system and objective tenderness in electrically stimulated beef *longissimus dorsi* muscle. *Meat Science, 58*(2), 167-174.

Ilian, M. A., Bekhit, A. E.-D., & Bickerstaffe, R. (2004a). The relationship between meat tenderization, myofibril fragmentation and autolysis of calpain 3 during post-mortem aging. *Meat Science, 66*(2), 387-397.

Ilian, M. A., Bekhit, A. E.-D. A., & Bickerstaffe, R. (2004b). Does the newly discovered calpain 10 play a role in meat tenderization during post-mortem storage? *Meat Science, 66*(2), 317-327.

Ilian, M. A., Bekhit, A. E.-D. A., Stevenson, B., Morton, J. D., Isherwood, P., & Bickerstaffe, R. (2004). Up- and down-regulation of longissimus tenderness parallels changes in the myofibril-bound calpain 3 protein. *Meat Science, 67*(3), 433-445.

Ilian, M. A., Bickerstaffe, R., & Greaser, M. L. (2004). Postmortem changes in myofibrillar-bound calpain 3 revealed by immunofluorescence microscopy. *Meat Science, 66*(1), 231-240.

Ishiura, S., Sugita, H., Nonaka, I., & Imahori, K. (1980). Calcium-activated neutral protease: its localization in the myofibril, especially at the z-band. *The Journal of Biochemistry, 87*(1), 343-346.

Ji, J. R., & Takahashi, K. (2006). Changes in concentration of sarcoplasmic free calcium during post-mortem ageing of meat. *Meat Science, 73*(3), 395-403.

Jiang, S., Wang, J., Chang, T., & Chen, C. (1997). A continuous method for measuring calpain activity. *Analytical Biochemistry, 244*(2), 233-238.

Kapprell, H. P., & Goll, D. E. (1989). Effect of Ca^{2+} on binding of the calpains to calpastatin*. *Journal of Biological Chemistry*, *264*(30), 17888-17896.

Kar, P., Samanta, K., Shaikh, S., Chowdhury, A., Chakraborti, T., & Chakraborti, S. (2010). Mitochondrial calpain system: An overview. *Archives of Biochemistry and Biophysics, 495*(1), 1-7.

Kemp, C. M., Bardsley, R. G., & Parr, T. (2006). Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle1. *Journal of Animal Science, 84*(10), 2841-2846.

Kemp, C. M., King, D. A., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2009). The caspase proteolytic system in callipyge and normal lambs in longissimus, semimembranosus, and infraspinatus muscles during postmortem storage1. *Journal of Animal Science, 87*(9), 2943-2951.

Kemp, C. M., & Parr, T. (2008). The effect of recombinant caspase 3 on myofibrillar proteins in porcine skeletal muscle. *Animal, 2*(8), 1254-1264.

Kemp, C. M., & Parr, T. (2012). Advances in apoptotic mediated proteolysis in meat tenderisation. *Meat Science, 92*(3), 252-259.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness – An enzymatic view. *Meat Science, 84*(2), 248-256.

Kendall, T. L., Koohmaraie, M., Arbona, J. R., Williams, S. E., & Young, L. L. (1993). Effect of pH and ionic strength on bovine m-calpain and calpastatin activity. *Journal of Animal Science, 71*(1), 96-104.

Khorchid, A., & Ikura, M. (2002). How calpain is activated by calcium. *Nature Structural Biology, 9*(4), 239-241.

Kim, Y. H., Lonergan, S. M., & Huff-Lonergan, E. (2010). Protein denaturing conditions in beef deep semimembranosus muscle results in limited μ-calpain activation and protein degradation. *Meat Science, 86*(3), 883-887.

Kim, Y. H. B., Stuart, A., Nygaard, G., & Rosenvold, K. (2012). High pre rigor temperature limits the ageing potential of beef that is not completely overcome by electrical stimulation and muscle restraining. *Meat Science, 91*(1), 62-68.

Kim, Y. H. B., Warner, R. D., & Rosenvold, K. (2014). Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: a review. *Animal Production Science, 54*(4), 375-395.

Koohmaraie, M. (1992). The role of Ca2+-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. *Biochimie, 74*(3), 239-245.

Koohmaraie, M. (1994). Muscle proteinases and meat aging. *Meat Science, 36*(1), 93-104.

Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Science, 43*, 193-201.

Koohmaraie, M., & Geesink, G. H. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science, 74*(1), 34-43.

Koohmaraie, M., Schollmeyer, J. E., & Dutson, T. R. (1986). Effect of low-calcium-requiring calcium activated factor on myofibrils under varying pH and temperature conditions. *Journal of Food Science, 51*(1), 28-32.

Koohmaraie, M., Seidemann, S. C., Schollmeyer, J. E., Dutson, T. R., & Crouse, J. D. (1987). Effect of post-mortem storage on Ca^{++}-dependent proteases, their inhibitor and myofibril fragmentation. *Meat Science, 19*(3), 187-196.

Koutakis, P., Miserlis, D., Myers, S. A., Kim, J. K. S., Zhu, Z., Papoutsi, E., Swanson, S. A., Haynatzki, G., Ha, D. M., Carpenter, L. A., McComb, R. D., Johanning, J. M., Casale, G. P., & Pipinos, I. I. (2015). Abnormal accumulation of desmin in gastrocnemius myofibers of patients with peripheral artery disease: Associations with altered myofiber morphology and density, mitochondrial dysfunction and impaired limb function. *Journal of Histochemistry & Cytochemistry, 63*(4), 256-269.

Kristensen, L., & Purslow, P. P. (2001). The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. *Meat Science, 58*(1), 17-23.

Lamare, M., Taylor, R. G., Farout, L., Briand, Y., & Briand, M. (2002). Changes in proteasome activity during postmortem aging of bovine muscle. *Meat Science, 61*(2), 199-204.

Lametsch, R., Lonergan, S., & Huff-Lonergan, E. (2008). Disulfide bond within μ-calpain active site inhibits activity and autolysis. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1784, 1215–1221.

Lana, A., & Zolla, L. (2016). Proteolysis in meat tenderization from the point of view of each single protein: A proteomic perspective. *Journal of Proteomics, 147*, 85-97.

Le Bihan-Duval, E., Debut, M., Berri, C. M., Sellier, N., Santé-Lhoutellier, V., Jégo, Y., & Beaumont, C. (2008). Chicken meat quality: genetic variability and relationship with growth and muscle characteristics. *BMC Genetics*, *9*(1), 53.

Li, X., Zhang, D., Ren, C., Bai, Y., Ijaz, M., Hou, C., & Chen, L. (2021). Effects of protein posttranslational modifications on meat quality: A review. *Comprehensive Reviews in Food Science and Food Safety*, *20*(1), 289-331.

Li, Y.-p., Liu, R., Zhang, W.-g., Fu, Q.-q., Liu, N., & Zhou, G.-h. (2014). Effect of nitric oxide on μ-calpain activation, protein proteolysis, and protein oxidation of pork during post-Mortem aging. *Journal of Agricultural and Food Chemistry*, *62*(25), 5972-5977.

Liu, J., Ruusunen, M., Puolanne, E., & Ertbjerg, P. (2014). Effect of pre-rigor temperature incubation on sarcoplasmic protein solubility, calpain activity and meat properties in porcine muscle. Lwt-Food Science and Technology, 55(2), 483-489.

Liu, P., Zhang, Z., Guo, X., Zhu, X., Mao, X., Guo, X., . . . Zhang, J. (2021). μ-Calpain oxidation and proteolytic changes on myofibrillar proteins from Coregonus Peled in vitro. *Food Chemistry*, *361*, 130100.

Liu, R., Li, Y., Wang, M., Zhou, G., & Zhang, W. (2016). Effect of protein S-nitrosylation on autolysis and catalytic ability of μ-calpain. *Food Chemistry, 213*, 470-477.

Liu, R., Lonergan, S., Steadham, E., Zhou, G., Zhang, W., & Huff-Lonergan, E. (2019a). Effect of nitric oxide and calpastatin on the inhibition of μ-calpain activity, autolysis and proteolysis of myofibrillar proteins. *Food Chemistry*, *275*, 77-84.

Liu, R., Lonergan, S., Steadham, E., Zhou, G., Zhang, W., & Huff-Lonergan, E. (2019b). Effect of nitric oxide on myofibrillar proteins and the susceptibility to calpain-1 proteolysis. *Food Chemistry*, *276*, 63-70.

Liu, R., Warner, R. D., Zhou, G., & Zhang, W. (2018). Contribution of nitric oxide and protein S-nitrosylation to variation in fresh meat quality. *Meat Science*, *144*, 135-148.

Lonergan, S. M., Johnson, M. H., & Calkins, C. R. (1995). Improved calpain assay using fluorescein isothiocyanate‐labeled casein. *Journal of Food Science, 60*(1), 72-73.

López-Bote, C. (2017). Chapter 4 - Chemical and biochemical constitution of muscle. In F. Toldra (Ed.), *Lawrie´s Meat Science (Eighth Edition)* (pp. 99-158): Woodhead Publishing.

Lu, H.-T., Feng, R.-Q., Tang, J.-K., Zhou, J.-J., Gao, F., & Ren, J. (2020). CaMKII/calpain interaction mediates ischemia/reperfusion injury in isolated rat hearts. *Cell Death & Disease*, *11*(5), 388.

Lu, X., Zhang, Y., Xu, B., Zhu, L., & Luo, X. (2020). Protein degradation and structure changes of beef muscle during superchilled storage. *Meat Science, 168*, 108180.

Ma, D., & Kim, Y. H. B. (2020). Proteolytic changes of myofibrillar and small heat shock proteins in different bovine muscles during aging: Their relevance to tenderness and water-holding capacity. *Meat Science, 163*, 108090.

Maki, M., Takano, E., Mori, H., Sato, A., Murachi, T., & Hatanaka, M. (1987). All four internally repetitive domains of pig calpastatin possess inhibitory activities against calpains I and II. *FEBS Letters*, *223*(1), 174-180.

Mellgren, R. L. (1987). Calcium-dependent proteases: an enzyme system active at cellular membranes? *The FASEB Journal, 1*(2), 110-115.

Mellgren, R. L., Mericle, M. T., & Lane, R. D. (1986). Proteolysis of the calcium-dependent protease inhibitor by myocardial calcium-dependent protease. *Archives of Biochemistry and Biophysics*, *246*(1), 233-239.

Melody, J. L., Lonergan, S. M., Rowe, L. J., Huiatt, T. W., Mayes, M. S., & Huff-Lonergan, E. (2004). Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. *Journal of Animal Science, 82*(4), 1195-1205.

Michetti, M., Salamino, F., Tedesco, I., Averna, M., Minafra, R., Melloni, E., & Pontremoli, S. (1996). Autolysis of human erythrocyte calpain produces two active enzyme forms with different cell localization. *FEBS Letters*, *392*(1), 11-15.

Milner, D. J., Mavroidis, M., Weisleder, N., & Capetanaki, Y. (2000). Desmin cytoskeleton linked to muscle mitochondrial distribution and respiratory function. *Journal of Cell Biology, 150*(6), 1283-1298.

Milton, D. L., Norqvist, A., & Wolfwatz, H. (1992). Cloning of a metalloprotease gene involved in the virulence mechanism of Vibrio anguillarum. *Journal of Bacteriology, 174*(22), 7235-7244.

Mohrhauser, D. A., Lonergan, S. M., Huff-Lonergan, E., Underwood, K. R., & Weaver, A. D. (2014). Calpain-1 activity in bovine muscle is primarily influenced by temperature, not pH decline. *Journal of Animal Science, 92*(3), 1261-1270.

Mohrhauser, D. A., Underwood, K. R., & Weaver, A. D. (2011). In vitro degradation of bovine myofibrils is caused by μ-calpain, not caspase-3. *Journal of Animal Science, 89*(3), 798-808.

Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z., & Davies, P. L. (2002). A Ca2+ switch aligns the active site of calpain. *Cell, 108*(5), 649-660.

Molinari, M., & Carafoli, E. (1997). Calpain: a cytosolic proteinase active at the membranes. *The Journal of Membrane Biology, 156*(1), 1-8.

Momeni, H. R. (2011). Role of calpain in apoptosis. *Cell journal, 13*(2), 65-72.

Moshal, K. S., Singh, M., Sen, U., Rosenberger, D. S. E., Henderson, B., Tyagi, N., Zhang, H. & Tyagi, S. C. (2006). Homocysteine-mediated activation and mitochondrial translocation of calpain regulates MMP-9 in MVEC. *American Journal of Physiology-Heart and Circulatory Physiology, 291*(6), H2825-H2835.

Mungure, T. E., Bekhit, A. E.-D. A., Birch, E. J., & Stewart, I. (2016). Effect of rigor temperature, ageing and display time on the meat quality and lipid oxidative stability of hot boned beef Semimembranosus muscle. *Meat Science, 114*, 146-153.

Nagaraj, N. S., Anilakumar, K. R., & Santhanam, K. (2002). Changes in the calpain-calpastatin and cathepsin (B, B+L, H and D) during postmortem storage of goat muscles. *Journal of Food Biochemistry, 26*(1), 75-89.

Neath, K. E., Del Barrio, A. N., Lapitan, R. M., Herrera, J. R. V., Cruz, L. C., Fujihara, T., Muroya, S., Chikuni, K., Hirabayashi, M. & Kanai, Y. (2007). Protease activity higher in postmortem water buffalo meat than Brahman beef. *Meat Science, 77*(3), 389-396.

Ni, R., Zheng, D., Wang, Q., Yu, Y., Chen, R., Sun, T., . . . Peng, T. (2015). Deletion of capn4 protects the heart against endotoxemic injury by preventing ATP synthase disruption and inhibiting mitochondrial superoxide generation. *Circulation: Heart Failure*, *8*(5), 988-996.

Offer, G. (1991). Modelling of the formation of pale, soft and exudative meat: Effects of chilling regime and rate and extent of glycolysis. *Meat Science, 30*(2), 157-184.

Offer, G., & Cousins, T. (1992). The mechanism of drip production: Formation of two compartments of extracellular space in muscle Post mortem. *Journal of the Science of Food and Agriculture*, *58*(1), 107-116.

Offer, G., & Knight, P. (1988). Structural basis of WHC in meat. Part 2: Drip losses. In R. Lawrie (Ed.). Developments in meat science (pp. 173-243). London: Elsevier Applied Science.

Offer, G., Knight, P., Jeacocke, R., Almond, R., Cousins, T., Elsey, J., Parsons, N., Sharp, A., Starr, R., & Purslow, P. (1989). The structural basis of the water-holding, appearance and toughness of meat and meat products. *Food Microstructure, 8*(1), 151-170.

Offer, G., & Trinick, J. (1983). On the mechanism of water holding in meat: The swelling and shrinking of myofibrils. *Meat Science, 8*(4), 245-281.

Ouali, A. (1990). Meat tenderization: possible causes and mechanisms. A review. *Journal of Muscle Foods, 1*(2), 129-165.

Ozaki, T., Tomita, H., Tamai, M., & Ishiguro, SI. (2007). Characteristics of Mitochondrial Calpains. *The Journal of Biochemistry, 142*(3), 365-376.

Ozaki, T., Yamashita, T., & Ishiguro, S.-i. (2008). ERp57-associated mitochondrial μ-calpain truncates apoptosis-inducing factor. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1783*(10), 1955-1963.

Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric Oxide and Peroxynitrite in Health and Disease. *Physiological Reviews*, *87*(1), 315-424.

Parrish JR., F. C., Selvig, C. J., Culler, R. D., & Zeece, M. G. (1981). CAF activity, calcium concentration, and the 30,000-dalton component of tough and tender bovine longissimus muscle. *Journal of Food Science*, *46*(1), 308-311.

Pearce, K. L., Rosenvold, K., Andersen, H. J., & Hopkins, D. L. (2011). Water distribution and mobility in meat during the conversion of muscle to meat and ageing and the impacts on fresh meat quality attributes - A review. *Meat Science, 89*(2), 111-124.

Penny, I. F. (1967). The influence of pH and temperature on the properties of myosin. *The Biochemical journal, 104*(2), 609-615.

Perrin, B. J., & Huttenlocher, A. (2002). Calpain. *The International Journal of Biochemistry & Cell Biology, 34*(7), 722-725.

Peter, A. K., Cheng, H., Ross, R. S., Knowlton, K. U., & Chen, J. (2011). The costamere bridges sarcomeres to the sarcolemma in striated muscle. *Progress in Pediatric Cardiology*, *31*(2), 83-88.

Phelps, K. J., Drouillard, J. S., Silva, M. B., Miranda, L. D. F., Ebarb, S. M., Van Bibber-Krueger, C. L., O'Quinn, T. G. & Gonzalez, J. M. (2016). Effect of extended postmortem aging and steak location on myofibrillar protein degradation and Warner-Bratzler shear force of beef M. semitendinosus steaks. *Journal of Animal Science, 94*(1), 412-423.

Pomponio, L., & Ertbjerg, P. (2012). The effect of temperature on the activity of μ- and m-calpain and calpastatin during post-mortem storage of porcine longissimus muscle. *Meat Science, 91*(1), 50-55.

Pomponio, L., Lametsch, R., Karlsson, A. H., Costa, L. N., Grossi, A., & Ertbjerg, P. (2008). Evidence for post-mortem m-calpain autolysis in porcine muscle. *Meat Science, 80*(3), 761-764.

Puolanne, E., & Halonen, M. (2010). Theoretical aspects of water-holding in meat. *Meat Science*, *86*(1), 151-165.

Purslow, P. P. (2017). Chapter 3 - The Structure and Growth of Muscle. In F. Toldra (Ed.), *Lawrie´s Meat Science (Eighth Edition)* (pp. 49-97): Woodhead Publishing.

Qin, J., Deng, X., Lei, Y., Liu, P., Lu, S., & Zhang, J. (2020). Effects of μ-calpain oxidation on Coregonus peled myofibrillar protein degradation in vitro. *Journal of Food Science, 85*(3), 682-688.

Raser, K. J., Posner, A., & Wang, K. K. W. (1995). Casein zymography: a method to study μ-calpain, m-calpain, and their inhibitory agents. *Archives of Biochemistry and Biophysics, 319*(1), 211-216.

Rayment, I., Rypniewski, W. R., Schmidt-Bäse, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G. & Holden, H. M. (1993). Three-dimensional structure of myosin subfragment-1: A molecular motor. *Science, 261*(5117), 50-58.

Rios, F. J., Zou, Z.-G., Harvey, A. P., Harvey, K. Y., Nosalski, R., Anyfanti, P., . . . Touyz, R. M. (2020). Chanzyme TRPM7 protects against cardiovascular inflammation and fibrosis. *Cardiovascular Research*, *116*(3), 721-735.

Rowe, L. J., Maddock, K. R., Lonergan, S. M., & Huff-Lonergan, E. (2004a). Influence of early postmortem protein oxidation on beef quality. *Journal of Animal Science, 82*(3), 785-793.

Rowe, L. J., Maddock, K. R., Lonergan, S. M., & Huff-Lonergan, E. (2004b). Oxidative environments decrease tenderization of beef steaks through inactivation of μ-calpain. *Journal of Animal Science, 82*(11), 3254-3266.

Savage, A. W. J., Warriss, P. D., & Jolley, P. D. (1990). The amount and composition of the proteins in drip from stored pig meat. *Meat Science, 27*(4), 289-303.

Sentandreu, M. A., Coulis, G., & Ouali, A. (2002). Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends in Food Science & Technology, 13*(12), 400-421.

Shackelford, S. D., Wheeler, T. L., Meade, M. K., Reagan, J. O., Byrnes, B. L., & Koohmaraie, M. (2001). Consumer impressions of Tender Select beef. *Journal of Animal Science, 79*(10), 2605-2614.

Sogawa, K., & Takahashi, K. (1978). Use of fluorescamine-labeled casein as a substrate for assay of proteinases. *The Journal of Biochemistry, 83*(6), 1783-1787.

Sorimachi, H., Saido, T. C., & Suzuki, K. (1994). New era of calpain research. *FEBS Letters, 343*(1), 1-5.

Sorimachi, H., & Suzuki, K. (2001). The structure of calpain. *The Journal of Biochemistry, 129*(5), 653-664.

Spencer, M. J., & Tidball, J. G. (1996). Calpain translocation during muscle fiber necrosis and regeneration in dystrophin-deficient mice. *Experimental Cell Research, 226*(2), 264-272.

Takahashi, K. (1992). Non-enzymatic weakening of myofibrillar structures during conditioning of meat: calcium ions at 0.1 mM and their effect on meat tenderization. *Biochimie, 74*(3), 247-250.

Takahashi, K. (1996). Structural weakening of skeletal muscle tissue during post-mortem ageing of meat: the non-enzymatic mechanism of meat tenderization. *Meat Science, 43*, 67-80.

Taylor, R. G., Geesink, G. H., Thompson, V. F., Koohmaraie, M., & Goll, D. E. (1995). Is Z-disk degradation responsible for postmortem tenderization? *Journal of Animal Science, 73*(5), 1351-1367.

Thomas, A. R., Gondoza, H., Hoffman, L. C., Oosthuizen, V., & Naudé, R. J. (2004). The roles of the proteasome, and cathepsins B, L, H and D, in ostrich meat tenderisation. *Meat Science, 67*(1), 113-120.

Thompson, J., Hu, Y., Lesnefsky, E. J., & Chen, Q. (2016). Activation of mitochondrial calpain and increased cardiac injury: beyond AIF release. *American Journal of Physiology-Heart and Circulatory Physiology*, *310*(3), H376-H384.

Thompson, V. F., Saldaña, S., Cong, J., & Goll, D. E. (2000). A BODIPY Fluorescent Microplate Assay for Measuring Activity of Calpains and Other Proteases. *Analytical Biochemistry, 279*(2), 170-178.

Troeberg, L., & Nagase, H. (2003). Zymography of Metalloproteinases. *Current Protocols in Protein Science, 33*(1), 21.15.21-21.15.12.

Twining, S. S. (1984). Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Analytical Biochemistry, 143*(1), 30-34.

Wang, A., Kang, D., Zhang, W., Zhang, C., Zou, Y., & Zhou, G. (2018). Changes in calpain activity, protein degradation and microstructure of beef M. semitendinosus by the application of ultrasound. *Food Chemistry, 245*, 724-730.

Wang, J., Yan, X.-l., Liu, R., Fu, Q.-q., Zhou, G.-h., & Zhang, W.-g. (2016). Differences in calpain system, desmin degradation and water holding capacity between commercial Meishan and Duroc × Landrace × Yorkshire crossbred pork. *Animal Science Journal*, *87*(1), 109-116.

Wang, K. K. W. (2000). Calpain and caspase: can you tell the difference? *Trends in Neurosciences, 23*(1), 20-26.

Wang, L.-L., Han, L., Ma, X.-L., Yu, Q.-L., & Zhao, S.-N. (2017). Effect of mitochondrial apoptotic activation through the mitochondrial membrane permeability transition pore on yak meat tenderness during postmortem aging. *Food Chemistry*, *234*, 323-331.

Wang, L., Li, J., Teng, S., Zhang, W., Purslow, P. P., & Zhang, R. (2022). Changes in collagen properties and cathepsin activity of beef M. semitendinosus by the application of ultrasound during post-mortem aging. *Meat Science*, *185*, 108718.

Warner, R. D., Kerr, M., Kim, Y. H. B., & Geesink, G. (2014). Pre-rigor carcass stretching counteracts the negative effects of high rigor temperature on tenderness and water-holding capacity – using lamb muscles as a model. *Animal Production Science, 54*(4), 494-503.

Veiseth, E., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2001). Effect of postmortem storage on μ-calpain and m-calpain in ovine skeletal muscle. *Journal of Animal Science, 79*(6), 1502-1508.

Whipple, G., Koohmaraie, M., Dikeman, M. E., Crouse, J. D., Hunt, M. C., & Klemm, R. D. (1990). Evaluation of attributes that affect longissimus muscle tenderness in bos taurus and bos indicus cattle. *Journal of Animal Science*, *68*(9), 2716-2728.

Whiting, R. C. (1980). Calcium uptake by bovine muscle mitochondria and sarcoplasmic reticulum. *Journal of Food Science, 45*(2), 288-292.

Vosler, P. S., Brennan, C. S., & Chen, J. (2008). Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. *Molecular Neurobiology, 38*(1), 78-100.

Wulf, D. M., O'Connor, S. F., Tatum, J. D., & Smith, G. C. (1997). Using objective measures of muscle color to predict beef longissimus tenderness1. *Journal of Animal Science*, *75*(3), 684-692.

Yang, Y., Han, L., Yu, Q., Gao, Y., & Song, R. (2020). Study of the AMP-activated Protein Kinase Role in Energy Metabolism Changes during the Postmortem Aging of Yak Longissimus dorsal. *Animals*, *10*(3), 427.

Zatz, M., & Starling, A. (2005). Calpains and disease. *New England Journal of Medicine*, *352*(23), 2413-2423.

Zeng, Z., Li, C., & Ertbjerg, P. (2017). Relationship between proteolysis and water-holding of myofibrils. *Meat Science, 131*, 48-55.

Zhang, C., Liu, R., Wang, A., Kang, D., Zhou, G., & Zhang, W. (2018). Regulation of calpain-1 activity and protein proteolysis by protein nitrosylation in postmortem beef. *Meat Science, 141*, 44-49.

Zhang, J., Li, M., Yu, Q., Han, L., & Ma, Z. (2019). Effects of Lysosomal–Mitochondrial Apoptotic Pathway on Tenderness in Post-Mortem Bovine Longissimus Muscle. *Journal of Agricultural and Food Chemistry*, *67*(16), 4578-4587.

Zhang, K., Cremers, M. M., Wiedemann, S., Poitz, D. M., Pfluecke, C., Heinzel, F. R., . . . Heidrich, F. M. (2021a). Spatio-temporal regulation of calpain activity after experimental myocardial infarction in vivo. *Biochemistry and Biophysics Reports*, *28*, 101162.

Zhang, M., Wang, G., & Peng, T. (2021b). Calpain-Mediated Mitochondrial Damage: An Emerging Mechanism Contributing to Cardiac Disease. *Cells*, *10*(8), 2024.

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

Zhang, Z., Liu, P., Deng, X., Guo, X., Mao, X., Guo, X., & Zhang, J. (2021c). Effects of hydroxyl radical oxidation on myofibrillar protein and its susceptibility to μ-calpain proteolysis. LWT, 137, 110453.

Zhu, X., Ruusunen, M., Gusella, M., Ylä-Ajos, M., Xu, X., Zhou, G., & Puolanne, E. (2013). High early post-mortem temperature induces activation of AMP-activated protein kinase and development of pale, soft and exudative characteristics in turkey muscles. *Meat Science, 93*(3), 600-606.

Zhu, X., Ruusunen, M., Gusella, M., Zhou, G., & Puolanne, E. (2011). High post-mortem temperature combined with rapid glycolysis induces phosphorylase denaturation and produces pale and exudative characteristics in broiler Pectoralis major muscles. *Meat Science, 89*(2), 181-188.