# Ca2+-induced binding of calpain-2 to myofibrils: Preliminary results in pork longissimus thoracis muscle supporting a role on myofibrillar protein degradation

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Meat Science

# Ca2+-induced binding of calpain-2 to myofibrils: Preliminary results in pork longissimus thoracis muscle supporting a role on myofibrillar protein degradation

--Manuscript Draft--

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| | Per Ertbjerg, PhD |
| **Abstract:** | The aim of this study was to investigate the role of $Ca^{2+}$ in the process of calpain-2 becoming associated to myofibrils and the potential of myofibril-bound calpain to degrade myofibrillar proteins. Different $Ca^{2+}$ concentrations were applied to myofibrils mixed with partially purified calpain-2. $Ca^{2+}$ induced binding of calpain to myofibrils in a concentration-dependent manner. The half-maximal $Ca^{2+}$ requirements for binding of calpain-2 to myofibrils and for calpain-2 proteolysis of myofibrils were 0.60 mM and 0.29 mM, respectively. To investigate the proteolytic activity of myofibril-bound calpain, a mixture of myofibrils and calpain-2 was briefly incubated with $Ca^{2+}$. Unbound calpain was removed by washing with a $Ca^{2+}$-free buffer. The myofibril-bound calpain maintained proteolytic activity and degraded desmin when re-activated with $Ca^{2+}$. In conclusion, the results suggest that an increase in $Ca^{2+}$ will activate and induce binding of calpain to myofibrils. Subsequently, calpain is relatively tightly bound and proteolytically active. |
| **Suggested Reviewers:** | S.M. Lonergan<br>slonerga@iastate.edu<br>Steven Lonergan is an expert on calpain biochemistry in meat. |
| | Tim Parr<br>tim.parr@nottingham.ac.uk<br>Tim Parr is an expert on the function of various proteolytic enzymes in muscle and meat. |
| **Response to Reviewers:** | |

**Cover letter** October 16th 2020

Dear Editor,

Enclosed is the revised manuscript which after the revision now is entitled "$Ca^{2+}$-induced binding of calpain-2 to myofibrils: Preliminary results in pork *longissimus thoracis* muscle supporting a role on myofibrillar protein degradation" which we hope will be considered for publication in Meat Science.

In the revision Table 1was replaced by a new figure to respond to the reviewers critisism. Furthermore, the last figure was removed.

The novelty of the present manuscript is that it provides information on the mechanism involved in binding of calpain-2 to myofibrils. In addition it shows that myofibril-bound calpain maintains proteolytic activity and degrades desmin.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the publication of this manuscript and, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

We upload in separate files the response to reviewers, the revised manuscript, six Figures and one Table.

Yours sincerely

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

**Thanks to the authors who considered one of the major concerns of the reviewer by including new results with calcium alone. However, the authors should mention that they only considered desmin in this study and further structural proteins are worthy to consider in the future to better understand the roles of both proteases or calcium alone or in combination.**
*Thanks for your advice. We have mentioned the suggested statement at the end of section 4.1.*

**Please, the figure 6 is not at all appropriate. The referee ask to remove it as well as the sentence 304-305. At this stage the preliminary results of this study don't allow take any conclusion or validate any model.**
*We have removed this figure and the sentence in the manuscript according to the suggestion.*

**Further, the title is not really telling us new insights. It should be changed to avoid confusion. Please, "preliminary results" should be emphasised in the title. The referee suggest the following "Ca2+-induced binding of calpain-2 to myofibrils: preliminary results in pork longissimus muscle supporting a role on myofibrillar protein breakdown".**
*The title has been changed as suggested.*

**It is not clear for the referee why the authors are discussing much more calpain 1 in their manuscript rather than calpain 2. Please, all the section 314 - 318 is not needed.**
*Deleted.*

**Last comment concerns the Table 1. Please, it is more appropriate to show these results using a graph. The title of the new figure needs also revision. A relationship should be characterised by an extent of link, please amend.**
*A new figure (Fig. 1) has been included instead of the data in Table 1 and the title revised.*

# $Ca^{2+}$-induced binding of calpain-2 to myofibrils: Preliminary results in pork *longissimus thoracis* muscle supporting a role on myofibrillar protein degradation

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## Abstract

The aim of this study was to investigate the role of $Ca^{2+}$ in the process of calpain-2 becoming associated to myofibrils and the potential of myofibril-bound calpain to degrade myofibrillar proteins. Different $Ca^{2+}$ concentrations were applied to myofibrils mixed with partially purified calpain-2. $Ca^{2+}$ induced binding of calpain to myofibrils in a concentration-dependent manner. The half-maximal $Ca^{2+}$ requirements for binding of calpain-2 to myofibrils and for calpain-2 proteolysis of myofibrils were 0.60 mM and 0.29 mM, respectively. To investigate the proteolytic activity of myofibril-bound calpain, a mixture of myofibrils and calpain-2 was briefly incubated with $Ca^{2+}$. Unbound calpain was removed by washing with a $Ca^{2+}$-free buffer. The myofibril-bound calpain maintained proteolytic activity and degraded desmin when re-activated with $Ca^{2+}$. In conclusion, the results suggest that an increase in $Ca^{2+}$ will activate and induce binding of calpain to myofibrils. Subsequently, calpain is relatively tightly bound and proteolytically active.

## Key words

Myofibril-bound calpain, Calcium, Desmin, Pork

## 1. Introduction

Calpains belong to a $Ca^{2+}$ dependent protease system which is believed to play an important role in meat tenderization (Huff Lonergan, Zhang, & Lonergan, 2010). The most studied are the two ubiquitous calpains, calpain-1 and calpain-2, also known as μ- and m-calpain, respectively. $Ca^{2+}$ concentration in the sarcoplasm, the inhibitor calpastatin autolysis, phosphorylation and its intracellular location regulates the activity of calpain (Perrin & Huttenlocher, 2002). In postmortem muscle, the extractable activity of calpain-1 declines substantially during storage, however, calpain-2 activity is relatively more stable (Boehm, Kendall, Thompson, & Goll, 1998; Koohmaraie, Seidemann, Schollmeyer, Dutson, & Crouse, 1987; Veiseth, Shackelford, Wheeler, Koohmaraie, & Hruska, 2001). It is widely accepted that calpain-1 contributes to proteolysis of myofibrillar proteins and meat tenderization during cold storage (Huff Lonergan et al., 2010; Kemp & Parr, 2012), whereas the contribution of calpain-2 is still debated. However, in pork it has been observed that calpain-2 is partly autolyzed during cold storage (Pomponio, Lametsch, Karlsson, Costa, Grossi, & Ertbjerg, 2008). The autolysis of calpain-2 is accelerated by higher muscle temperature early postmortem (Liu, Ruusunen, Puolanne, & Ertbjerg, 2014; Pomponio & Ertbjerg, 2012) and by the freezing-thawing process of pork (Zhang & Ertbjerg, 2018) and beef (Colle, Nasados, Rogers, Kerby, Colle, Van Buren, et al., 2018). Calpain-2 activation has also been reported during prolonged storage of beef (Camou, Marchello, Thompson, Mares, & Goll, 2007; Colle & Doumit, 2017; Phelps, Drouillard, Silva, Miranda, Ebarb, Van Bibber-Krueger, et al., 2016) and goat (Nagaraj, Anilakumar, & Santhanam, 2002). Taken together, these observations suggest a potential role for calpain-2 in postmortem proteolysis.

Calpains are located exclusively intracellularly and are predominantly free in sarcoplasm (Goll, Thompson, Li, Wei, & Cong, 2003), although part of the calpains are associated with subcellular organelles such as myofibrils (Ishiura, Sugita, Nonaka, & Imahori, 1980), mitochondria (Thompson, Hu, Lesnefsky, & Chen, 2016) and membranes (Hood, Logan, Sinai, Brooks, &

Roszman, 2003). In postmortem muscle, one key question yet to be resolved is the spatial distribution of calpains with postmortem storage and the mechanisms involved in translocation from the sarcoplasm. Few studies have reported that calpain-1 progressively binds to myofibrils during postmortem storage (Boehm et al., 1998; Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001; Melody, Lonergan, Rowe, Huiatt, Mayes, & Huff-Lonergan, 2004; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). It was reported that over 50% of total muscle calpain-1 was tightly bound to myofibrils at day 7 in beef (Boehm et al., 1998). Although a relatively large amount of calpain-1 bound to myofibrils, the activity of myofibril-bound calpain-1 against casein was very low compared with that of calpain-1 extracted from sarcoplasm. Similarly, Delgado et al. (2001) reported that in sheep longissimus muscle at day 1 around 40% of total calpain-1 was bound to myofibrils, however, the activity of myofibril-bound calpain against casein only accounted for 4% of the total calpain-1 activity. However, the myofibril-bound calpain showed some proteolytic activity against myofibrillar proteins (Delgado et al., 2001; Boehm et al., 1998). In agreement, Zeng, Li, & Ertbjerg (2017) found degradation of desmin in pork by myofibril-bound enzymes which were suggested to be calpain-1 and calpain-2. The observation that the amount of bound calpain-1 gradually increases with postmortem storage (Boehm et al., 1998; Melody et al., 2004) may provide insights into how calpain works during meat aging. However, there is very little available information about the nature of the binding of calpain-2 to myofibrils and the proteolytic activity of myofibril-bound calpain. Therefore, the aim of current study was to provide basic information on the role of $Ca^{2+}$ in calpain-2 becoming associated to myofibrils and to study how this process affects the activity of the myofibril-bound calpain.

## 2. Materials and methods

### *2.1. Sample processing*

Five pigs with a live weight of approximately 110 kg were slaughtered at a commercial slaughterhouse in Finland (HK-Ruokatalo, Forssa) and five pork loins from different animals were excised at 6 h post-mortem, vacuum-packaged, and transported on ice to the meat laboratory at University of Helsinki. The *longissimus thoracis* (LT) muscles were trimmed of visible connective tissue as well as fat. Then 120 – 150 g sample was cut from each muscle and this sample was further divided into several small parts (around 15 g each). All samples were vacuum-packaged at 12 h postmortem and frozen at -80 °C until use. The ultimate pH at 24 h postmortem was measured by an insertion electrode (Mettler-Toleda Inlab 427) and was for all muscles in the range of 5.4 to 5.6. Of the five muscle, two were used for calpain purification and the remaining three muscles were used for preparation of myofibrils. Before use, all muscle samples were placed in a 5 °C walk-in cold room to thaw for 2 hours and were then coarsely chopped. Myofibril preparations from these three muscles were subsequently incubated with calpain-2 to determine the effect of $Ca^{2+}$ on the binding of calpain-2 to myofibrils and the activity of myofibril-bound calpain.

*2.2. Preparation of myofibrils*

Myofibrils were prepared as described by Liu, Arner, Puolanne, & Ertbjerg (2016) with slight modifications. Briefly, meat samples were mixed 1:10 (w:v) with cold rigor buffer (75 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM EGTA, pH 7.0) and homogenized by an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany) at 13,500 rpm for 20 s. The supernatant was removed after centrifugation at 10,000 × *g* at 4 °C for 10 min and the pellet was then washed twice by incubation buffer (75 mM KCl, 100 mM Tris, 2 mM $MgCl_2$, pH 7.0) at 10,000 g at 4 °C for 5 min. The pellet was weighed and used directly in section 2.7 as the myofibril preparation, whereas the myofibrillar pellet was re-suspended in incubation buffer and diluted to 15 mg protein/mL for the experiments in sections 2.4, 2.5, 2.6 and 2.8. The protein concentration of each myofibril suspension was determined by the RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

99 *2.3. Purification of calpain-2*

100 Partial purification of calpain-2 was conducted according to the method described by Zeng et al.
101 (2017) with minor modifications. Muscle samples (around 120 g) were mixed with 600 mL cold
102 extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 10mM monothioglycerol, pH 8.3) and
103 homogenized at 13,500 rpm for 3 × 20 s. Then the homogenate was centrifuged at 10,000 × *g* at
104 4 °C for 30 min and the supernatant was filtered through cheesecloth to remove the connective
105 tissue and fat. The filtered extract was loaded onto a 26/10 DEAE Sepharose Fast Flow column (GE
106 Healthcare, Uppsala, Sweden) pre-equilibrated in buffer A (20 mM Tris-HCl, 5 mM EDTA and 10
107 mM monothioglycerol, pH 7.5) using a Pharmacia Biotech system® (AKTA prime) FPLC. Loading
108 and elution rate was 30 mL/min. The column was eluted using gradient of 0 to 0.6 M NaCl in buffer
109 A. Fractions eluted at 190 to 300 mM NaCl were collected and precipitated with 50% ammonium
110 sulfate. The precipitate was sedimented by centrifugation at 10,000 × *g* for 30 min and the pellet
111 was re-suspended in buffer A. This fraction was centrifuged again and filtered through a 0.45 μm
112 membrane before being loaded onto a Sephacryl S-300 HR 26/60 column (GE Healthcare) pre-
113 equilibrated with buffer A. Loading rate was 3 mL/min and elution rate was 5 mL/min. After that,
114 the fractions containing calpain activity was pooled, concentrated and purified by chromatography
115 on a 1 mL RESOURCE Q (GE Healthcare) with buffer A. The partial purified calpain-2 was
116 adjusted to contain 30% (v/v) glycerol, and the protein content was measured by RC DC Protein
117 Assay Kit before storage at -80 °C until use. The calpain-2 preparation showed no cross-reactivity
118 with calpain-1 when checked with western blot. Calpain-2 activity was determined by the casein
119 assay described by Ertbjerg, Henckel, Karlsson, Larsen, & Møller (1999) with minor modification.
120 Calpain-2 fractions were diluted in buffer A to appropriate concentrations to make the activity in
121 the linear region of the assay, and 100 μL of the diluted sample was incubated with 300 μL
122 incubation medium (100 mM Tris-HCl, 10 mM monothioglycerol, 5 mg/mL casein and 5 mM
123 $CaCl_2$, pH 7.5). After 30 min incubation at 25 °C, the reaction was stopped by 400 μL of 10%

trichloroacetic acid and then centrifuged at 20,000 × *g* for 5 min. Results were corrected by blanks with the same content incubated 0 min. One unit (U) of calpain activity was defined as an increase in absorbance at 278 nm of 1.0 per hour at 25 °C.

### *2.4. Measurement of free $Ca^{2+}$ concentration*

$Ca^{2+}$-binding proteins in myofibrils may absorb some $Ca^{2+}$ and thereby affect the free $Ca^{2+}$ concentration in a myofibril suspension. Therefore, the free $Ca^{2+}$ concentration in myofibril suspensions was determined by a $Ca^{2+}$ ion selective electrode equipped with a reference electrode (perfectION™ Combination $Ca^{2+}$ Electrode, Mettler Toledo AG, Greifensee, Switzerland). The measurement range of the electrode was from 0.5 μM to 1.0 M. The $Ca^{2+}$ concentration and millivolts were correlated by establishing a calibration curve before each run. Isolated myofibrils were dispersed in incubation buffer and the final protein concentration was adjusted to 15 mg/mL. To establish the relationship between added $CaCl_2$ and measured free $Ca^{2+}$, myofibril suspensions (4 mL) were mixed with 105 μL of 4 M KCl and added various $CaCl_2$ concentrations before they were measured (Fig. 1). All measurements were performed in duplicates at room temperature. The measured free $Ca^{2+}$ concentration was lower than the added amount of $Ca^{2+}$, especially in the lower concentration range. Fig. 1 was used to obtain the free $Ca^{2+}$ concentration needed for binding of calpain-2 to myofibrils and for calpain-2 proteolysis of myofibrils.

### *2.5. Effect of $Ca^{2+}$ on proteolytic activity of calpain-2 against myofibrils*

Myofibrils were used as substrate for determination of the free $Ca^{2+}$ concentration required for proteolytic activity of calpain-2. The relationship between added amount of calpain-2 and rate of proteolysis of myofibrils was investigated to establish the linear region of calpain-2 activity. Different amounts of calpain-2 (0, 0.2, 0.4, 0.8 and 1.2 U in 40 μL, respectively) was added to 500 μL of myofibril suspension (15 mg protein/mL) in incubation buffer (75 mM KCl, 100 mM Tris, 2

mM $MgCl_2$, pH 7.0); then 5.0 mM of $CaCl_2$ was added and the mixture (final volume 550 μL) was incubated for 30 min at 25 °C. To illustrate the binding mechanism and to ensure maximum activation of calpain-2, a relative high concentration of 5 mM $Ca^{2+}$ was used in this part of the study. The reaction was stopped by 260 μL of EGTA in incubation buffer (20 mM EGTA, 75 mM KCl, 100 mM Tris, 2 mM $MgCl_2$, pH 7.0). For the background, calpain-2 was substituted by the same volume of incubation buffer. The mixture was centrifuged at 20,000 × *g* for 5 min and the absorbance of the supernatant was measured at 278 nm. The principle of this assay is that calpain-2 will cleave myofibrillar substrates and resultant soluble peptides are subsequently measured in the supernatant due to their content of aromatic amino acids giving rise to absorbance at 278 nm. The activity of calpain-2 on myofibrils was defined as the absorbance difference between the background and the sample with addition of calpain-2. Calpain-2 amounts larger than 0.2 U were outside the linear region (Fig. 2), and hence 0.2 U of calpain-2 was chosen in the following assay to determine the free $Ca^{2+}$ concentration required for proteolytic activity.

To determine the effect of the free $Ca^{2+}$ concentration on the proteolytic activity of calpain-2 against myofibrils, calpain-2 (0.2 U in 40 μL) was added to a myofibril suspensions (500 μL, 15 mg protein/mL) followed by addition of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 5.0 mM of $CaCl_2$ (final volume 550 μL). After 30 min incubation, the reaction was stopped by addition of 260 μL of 20 mM EGTA. Calpain-2 was substituted by the same volume of incubation buffer in each $Ca^{2+}$ concentration as the background. The mixture was centrifuged at 20,000 × *g* for 5 min and the activity of calpain-2 against myofibrils was measured and calculated as described above.

### *2.6. Effect of $Ca^{2+}$ on the ratio of calpain-2 bound to myofibrils*

In this assay calpain bound to myofibrils will be removed from the supernatant, whereas unbound calpain is measured as soluble activity. Calpain-2 (1.6 U in 40 μL incubation buffer) was added into

myofibril suspensions (500 μL, 15 mg protein/mL) and incubated for 5 min. Then 10 μL of $CaCl_2$ solution in incubation buffer was added to obtain a final concentration of 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 5.0 mM of $Ca^{2+}$, where $CaCl_2$ was substituted by the same volume of EGTA solution (10 mM EGTA, 75 mM KCl, 50 mM Tris, 2 mM $MgCl_2$, pH 7.0) in the control (0 $Ca^{2+}$). Suspensions were mixed and immediately centrifuged at 20,000 × *g* for 2 min. The calpain-2 activity remaining in the supernatant was measured by the casein assay described in section 2.3. The ratio of calpain-2 bound to myofibrils were calculated as:

$$\text{Ratio} = (a - b) / a \times 100\%$$

where a and b are the calpain-2 activity remaining in the supernatant without (a) and with (b) a brief exposure of the myofibril suspension to $Ca^{2+}$.

*2.7. Activity of myofibril-bound calpain-2*

To learn more about the nature of the $Ca^{2+}$-activated proteases bound to the myofibrils, three different treatments were conducted. First, the activity of endogenous $Ca^{2+}$-activated enzymes of the myofibril preparations were determined (treatment A). Myofibrils (50 mg myofibril pellet) were re-suspended in 200 μL incubation buffer (20 mM EGTA, 75 mM KCl, 100 mM Tris, 2 mM $MgCl_2$, pH 7.0) containing 5 mM of $CaCl_2$ and incubated for 0, 0.5, 1 and 2 h at 22 °C; then 150 μL of 10 mM EGTA solution was added to stop the reaction. In the next two treatments, myofibrils were mixed with exogenous calpain-2 (partial purified calpain-2) and binding of calpain was either not induced (addition of EGTA, treatment B) or induced (addition of 5 mM $CaCl_2$, treatment C) by $Ca^{2+}$. Thereafter, the myofibrils were washed to remove calpain-2 which had not bound to myofibrils after $Ca^{2+}$ or EGTA treatment. Next the myofibrils were incubated with 5 mM $CaCl_2$ in order to observe desmin degradation as an indicator of the proteolytic activity of myofibril-bound

calpain. To be specific, in treatment B and C: myofibrils (50 mg myofibril pellet) were resuspended in 200 μL incubation buffer containing 0.4 U of calpain-2 and incubated for 5 min at 22 °C. Then EGTA (treatment B) or $CaCl_2$ (treatment C) was added to a final concentration of 1 mM and 5 mM, respectively, and the tubes were thoroughly mixed and immediately centrifuged at 20,000 × *g* at room temperature for 2 min. Then the myofibril pellet was washed with one mL incubation buffer and centrifuged at 20,000 × *g* for 5 min. Thereafter, the washed myofibrils were incubated 0, 0.5, 1 and 2 h with 5 mM $CaCl_2$ to observe desmin degradation. The reaction was stopped by 150 μL of 10 mM EGTA. All myofibril pellets were processed for western blot analysis.

*2.8. Effect of $Ca^{2+}$ on the activity of myofibril-bound calpain-2*

Calpain-2 (0.4 U) was added into 200 μl of myofibril suspension (15 mg protein/mL) and incubated for 5 min at room temperature. Then $CaCl_2$ was added to a final concentration of 0, 0.4, 0.8, 1.6, 3.2 and 5 mM, resulting in a free $Ca^{2+}$ concentrations in the myofibril suspension of 0, 0.1, 0.4, 0.9, 2.4 and 4.2 mM, respectively, as measured in method 2.4. The tubes were mixed and immediately centrifuged at 20,000 × *g* at room temperature for 2 min. Then the myofibril pellet was washed with one mL incubation buffer and centrifuged at 20,000 × *g* for 5 min. Thereafter, the washed myofibrils were incubated with 0, 0.4, 0.8, 1.6, 3.2 and 5 mM added $Ca^{2+}$ at room temperature for 2 h. The reaction was stopped by 80 μL of 20 mM EGTA. In the control group, calpain-2 was substituted by same volume of incubation buffer, and 5 mM $Ca^{2+}$ was added in the binding and incubation process. All myofibril pellets were processed for western blot analysis.

*2.9. SDS-PAGE and western blot*

SDS-PAGE and western blot was run according to the method described by Zeng et al. (2017) with slight modification. After adjusting the samples to the same protein concentration, 32 μL diluted sample was mixed with 12.5 μL NuPAGE® LDS Sample Buffer (4X) and 5 μL NuPAGE® Sample

Reducing Agent (10X) (Invitrogen, Carlsbad, CA). Then the mixture was heat treated at 70 °C for 10 min and applied onto NuPAGE Novex 12% Bis-Tris gels (Invitrogen). Gels were settled in XCell SureLock® Mini-Cell electrophoresis chamber and the electrophoresis was run at 200 V for 50 min.

After electrophoresis, proteins in gels were transferred to Immun-Blot® PVDF Membranes (Bio-Rad Laboratories, Hercules, CA) in XCell II™ Blot Module with NuPAGE® Transfer Buffer (20X) from Invitrogen. The blotting process was performed for 1 h at 30 V. After blotting, membranes were blocked for 1 h in 20 mL of TBS (50 mM Tris, 150 mM NaCl, pH 7.5) with 50 g/L skim milk powder at room temperature. Then membranes were washed in TBST (50 mM Tris, 150 mM NaCl, 0.5 g/L Tween-20, pH 7.5) for 10 min. Membranes were then incubated with 15 μL mouse monoclonal anti-desmin antibody clone DE-U-10 (Invitrogen, Carlsbad, CA) in 15 mL TBST at room temperature for 1 h. Afterward, membranes were washed three times in TBST for 5-10 min and then incubated with 4 μL IRDye® 800 CW Donkey anti-mouse IgG (H+L) in 15 mL TBST with 20 g/L skim milk powder at room temperature for 1 h. Membranes were then washed in TBST for 5 min twice and in TBS for 5 min once. After washing, membranes were scanned by Odyssey Infrared Imaging System-CLx (LI-Cor Cop, Lincoln, NE) using the 800 nm channel.

*2.10. Statistical analysis*

Muscle number was defined as a random factor. For each muscle, four independent replicates were performed for the proteolytic activity of calpain-2 against myofibrils. Duplicates were done for each muscle for measuring the free $Ca^{2+}$ concentration in myofibril suspensions, for determining the binding ratio of calpain-2 to myofibrils, and the activity of myofibril-bound calpain-2. Data analysis was carried out using the IBM SPSS Statistics 25 software.

## 3. Results

### *3.1. Effect of free $Ca^{2+}$ on activity of calpain-2 and binding of calpain-2 to myofibrils*

Proteolytic activity of calpain-2 against myofibrils increased with the increase of free $Ca^{2+}$ concentration (Fig. 3A). The activity increased rapidly with $Ca^{2+}$ concentration up to 0.9 mM, and then increased gradually to reach a maximum around 2.4 mM free $Ca^{2+}$. Although this is a $Ca^{2+}$ value much higher than the average in postmortem muscle sarcoplasm, it does come into range of the ionized level in serum, typically reported as 1.3 mM. The effect of free $Ca^{2+}$ on the ratio of calpain-2 being bound to myofibrils showed a similar increasing trend compared to that of calpain-2 activity (Fig. 3B). Maximum binding occurred when the free $Ca^{2+}$ concentration reached 2.4 mM with around 60% of the added calpain being bound to myofibrils. The free $Ca^{2+}$ concentrations needed for half-maximal calpain-2 activity and half-maximal binding of calpain-2 to myofibrils were 0.29 and 0.60 mM, respectively. The data suggests that the process of calpain becoming bound to myofibrils is strongly linked to the process of activation by the $Ca^{2+}$ ion.

### *3.2. Activity of myofibril-bound calpain-2*

Desmin is a known substrate of calpain-2 and its degradation has often been used as a marker of calpain activity in postmortem muscle. Therefore, we analyzed desmin degradation to evaluate the activity of myofibril-bound calpain in this study. Desmin degradation products gradually accumulated during a 2 h incubation of purified myofibrils with 5 mM $Ca^{2+}$ (Fig. 4A), demonstrating the presence of endogenous $Ca^{2+}$-activated proteases in the isolated myofibrils. Next, myofibrils were mixed with partial purified calpain-2 to allow $Ca^{2+}$-induced binding, followed by washing off unbound calpain before a 2 h incubation with 5 mM added $Ca^{2+}$. Myofibrils mixed with calpain and not briefly exposed to $Ca^{2+}$ before washing of myofibrils did not produce further desmin degradation (Fig 4B). However, a brief exposure to 5 mM added $Ca^{2+}$ induced binding of calpain-2 to myofibrils that after washing and re-incubation of myofibrils with $Ca^{2+}$ resulted in

extensive desmin degradation (Fig. 4C). $Ca^{2+}$ addition without addition of calpain-2 only resulted in slight degradation of desmin (Fig. 5A). When calpain-2 was added in combination with $Ca^{2+}$, desmin degradation increased with the increase of $Ca^{2+}$ up to a free $Ca^{2+}$ concentration of 2.4 mM (Fig. 5B). At the lowest tested concentration of 0.1 mM free $Ca^{2+}$ in the binding and incubation process, the desmin degradation was evidently more than that without $Ca^{2+}$ addition. Almost no intact desmin remained at 0.9 mM free $Ca^{2+}$. The data demonstrate that myofibril-bound calpain-2 possesses proteolytic activity within the free $Ca^{2+}$ concentrations existing in postmortem muscle, and gives further support to the suggestion that the process of calpain becoming bound to myofibrils is strongly linked to the process of activation by the $Ca^{2+}$ ion.

### *3.3. Stability of myofibril-bound calpain-2*

Free or loosely bound calpain is presumably easy to wash off myofibrils. The effect of washing on the stability of myofibril-bound calpain-2 is illustrated in Fig. 6. The amount of desmin degradation products decreased gradually with increased number of washing-times. There was no intact desmin following one wash and 2 h incubation. Some intact desmin remained after two or four times wash, however, there was still sufficient proteolytically active myofibril-bound calpain-2 to partly degrade desmin.

## 4. Discussion

### *4.1. Hypothesis for the mechanism of myofibril-bound calpain-2 on degradation of structural proteins*

In the present study, we have shown that $Ca^{2+}$ induces the binding of calpain-2 to myofibrils and that the binding requires a free $Ca^{2+}$ concentration in the same range as that required for proteolytic activity (Fig. 3). The myofibril-bound calpain-2 was proteolytically active and able to degrade the myofibrillar protein desmin (Fig. 4). Desmin degradation caused by myofibril-bound calpain-2

299 increased with the increase of $Ca^{2+}$ (Fig. 5), and was evident at the level of free $Ca^{2+}$ of 0.4 mM as
300 has been reported in postmortem pork muscle (Pomponio & Ertbjerg, 2012; Zhang & Ertbjerg,
301 2018). The association between exogenous calpain-2 and myofibrils was relatively tight (Fig. 6).
302 Few other studies have reported that a part of the endogenous calpain-1 in the muscle fiber binds to
303 myofibrils (Boehm et al., 1998; Delgado et al., 2001; Ilian, Bekhit, Stevenson, Morton, Isherwood,
304 & Bickerstaffe, 2004; Rowe et al., 2004). Some studies have observed that the extractable activity
305 of calpain-2 decreased to some extent during postmortem storage (Boehm et al., 1998; Camou et al.,
306 2007; Phelps et al., 2016). . Once calpain is activated by an increased postmortem $Ca^{2+}$
307 concentration in the sarcoplasm, it will become associated with calpastatin and/or autolyze leading
308 to instability (Li, Thompson, & Goll, 2004). Loss of extractable activity can also be hypothesized to
309 be due to the binding of activated calpain to structural proteins within the myofibrils and binding to
310 other subcellular organelles (such as the sarcolemma, mitochondria and the sarcoplasmic
311 reticulum). The observation in the present study that myofibril-bound calpain can degrade structural
312 proteins in the presence of $Ca^{2+}$ (Fig. 4 and 5) is in agreement with Zeng et al. (2017) who reported
313 desmin degradation in pork by endogenous myofibril-bound proteases when isolated myofibrils
314 were incubated with various $Ca^{2+}$ concentrations intended to activate either calpain-1 only or
315 calpain-1 and calpain-2 combined. It should be noted, however, that the highest $Ca^{2+}$ concentration
316 of 5 mM used in the present study is probably never reached even locally in postmortem muscle. In
317 contrary to calpain-1, the contribution of calpain-2 to postmortem proteolysis will likely not reach
318 its full potential as it is limited by the reached $Ca^{2+}$ level. Calpain-2 is generally activated later
319 postmortem when large amount of the calpastatin has lost its inhibitory activity (Boehm et al.,
320 1998), which may suggest that loss of extractable calpain-2 activity is mainly associated to the
321 process of calpain-2 binding to myofibrils. However, compared to calpain-1, proteolytic activity of
322 calpain-2 against myofibrillar proteins occurs later as the $Ca^{2+}$ concentration only later postmortem
323 would increase sufficiently to activate calpain-2, and there might be a delay between increased free

$Ca^{2+}$ level and the activation of calpain-2 (Zhang & Ertbjerg, 2018). In the present study we only investigated the degradation of desmin as an indicator of the activity of myofibril-bound calpain-2. Other structural proteins are also worthy to consider in the future to better understand the role of myofibril-bound calpain and the interaction with $Ca^{2+}$.

*4.2. Effect of $Ca^{2+}$ on activity of calpain-2 and binding of calpain-2 to myofibrils*

The level of free $Ca^{2+}$ influences the regulation of calpain (Goll et al., 2003). In the current study, the $Ca^{2+}$ requirement for half-maximal activity of calpain-2 by using myofibrils as substrate was 0.29 mM, while the half-maximal $Ca^{2+}$ requirements for binding of calpain-2 to myofibrils was 0.60 mM. Other studies have reported that 0.38 mM $Ca^{2+}$ (Barrett, Goll, & Thompson, 1991) and 1 mM $Ca^{2+}$ (Ceña, Jaime, Beitrán, & Roncalés, 1992) were required for half-maximal activity of calpain-2 using rabbit and lamb skeletal muscle myofibrils as substrate, respectively. Barrett, Goll, & Thompson (1991) reported that there was no significant difference in $Ca^{2+}$ concentrations required for half-maximal proteolysis among casein, myofibrils and other protein- or peptide-substrates. In general, the reported $Ca^{2+}$ concentration required for half-maximal calpain-2 activity is in the range of 0.4 - 0.8 mM, which is much higher than the free $Ca^{2+}$ concentration in living cells (Goll et al., 2003). However, it is possible that the free $Ca^{2+}$ concentration reach a level sufficient to partly activate calpain-2 in the later postmortem storage times. Several studies have measured the concentration of free $Ca^{2+}$ in the sarcoplasm of postmortem muscle. Parrish JR., Selvig, Culler, & Zeece (1981) found that the free $Ca^{2+}$ concentration ranged from 0.63 to 0.97 mM in 10- to 14-day postmortem bovine *longissimus* muscles. In ovine muscle, the free $Ca^{2+}$ concentration was reported to increase to 0.19 mM after 7 days cold storage (Geesink, Taylor, Bekhit, & Bickerstaffe, 2001). In chilled-storage pork, the free $Ca^{2+}$ concentration was reported to increase from 0.14 mM on day 1 to 0.40 mM on day 9; and in frozen-then-chilled storage, the free $Ca^{2+}$ concentration increased to 0.42

mM one day after thawing (Zhang & Ertbjerg, 2018). Calpain-2 started to bind to myofibrils once $Ca^{2+}$ was added in the suspension (Fig. 3B) and desmin degradation products caused by proteolysis of myofibril-bound calpain-2 can be observed at 0.1 mM free $Ca^{2+}$. Combined with the reported $Ca^{2+}$ concentrations in postmortem muscle, we speculate that calpain-2 activation and association to myofibrils is initiated as the level of $Ca^{2+}$ concentration gradually increases in muscle during storage.

*4.3. Stability of myofibril-bound calpain-2*

In order to test whether exogenous calpain-2 was tightly binding to myofibrils, the myofibrils were washed for a different number of times after $Ca^{2+}$ inducing the binding process. After several washes with a large excess of buffer the bound calpain-2 still retained sufficient activity to partly degrade desmin, although the overall activity tended to decline. The decreased amount of desmin degradation products (Fig. 6), indicates that the proteolytic activity of myofibril-bound calpain-2 gradually declined with increasing number of washing times. Nevertheless, these results suggest that calpain associated tighly to the myofibrils following activitaion with $Ca^{2+}$. Furthermore, endogenous myofibril-bound calpain or a $Ca^{2+}$ activated calpain-like enzyme, even after 6 times washing, was still associated to the myofibrils and after activation by $Ca^{2+}$ proteolytically active and able to degrade desmin and produce the same pattern of desmin degradation products as calpain-2 (results not shown). Delgado et al. (2001) similarly reported that calpain was still tightly bound to myofibrils after 11 times washing. It is uncertain if the decreased amount of degradation products (Fig, 6) is due to a reduced amount of myofibril-bound calpain-2 or if calpain-2 still was bound to the myofibrils and partly lost its activity during repeated washings.

## 5. Conclusions

*In vitro,* $Ca^{2+}$ induces exogenous calpain-2 to bind to myofibrils and the myofibril-bound calpain remains proteolytically active to degrade desmin. The half-maximal $Ca^{2+}$ requirements for binding of calpain-2 to myofibrils and for calpain-2 proteolysis of myofibrils were 0.60 mM and 0.29 mM, respectively, suggesting that the process of calpain binding to myofibrils is strongly linked to the activation process by the $Ca^{2+}$ ion. Following $Ca^{2+}$-induced binding, the association of calpain-2 with myofibrils is relatively tight. The results provide new insight on the role of myofibril-bound calpain activity on the degradation of structural proteins.

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473 Figure legends

474

475 **Fig. 1.** Measured free $Ca^{2+}$ concentration as affected by added $CaCl_2$ in a myofibril suspension with
476 a protein concentration of 15 mg/mL (solid line). The dashed line indicates the predicted value
477 without the presence of myofibrils.

478

479 **Fig. 2.** The relationship between added amount of calpain-2 and rate of proteolysis of myofibrils
480 illustrating the linear region. Means ± standard deviation are shown. $^{a-d}$Means having different
481 superscripts differ ($P<0.05$).

482

483 **Fig. 3.** The effect of free $Ca^{2+}$ concentration on (A) the rate of calpain-2 proteolysis of myofibrils
484 and (B) the ratio of calpain-2 binding to myofibrils. Bound activity was calculated as the amount of
485 calpain-2 activity removed from the supernatant of a myofibril suspension by $Ca^{2+}$ addition
486 followed by centrifugation to sediment myofibrils and any myofibril-bound calpain. The calpain-2
487 activity was measured in the supernatant using the casein assay. The binding ratio was defined as
488 0% when $Ca^{2+}$ was substituted by EGTA; and as 100% if there was no calpain-2 activity in the
489 supernatant. Means ± standard deviation are shown. $^{a-f}$Means having different superscripts differ
490 ($P<0.05$).

491

492 **Fig. 4.** Western blot of desmin after a brief exposure of myofibrils to calpain-2 and $Ca^{2+}$ followed
493 by isolation and incubation of the myofibrils with 5 mM added $Ca^{2+}$. MF: purified myofibrils; A:
494 purified myofibrils incubated with 5 mM added $Ca^{2+}$ for 0, 0.5, 1 and 2 h; B: myofibrils + calpain-2
495 + EGTA, washed and incubated with 5 mM added $Ca^{2+}$ for 0 and 2 h; C: myofibrils + calpain-2 +
496 $Ca^{2+}$, washed and incubated with 5 mM added $Ca^{2+}$ for 0, 0.5, 1 and 2 h. Each lane was loaded with
497 8 μg porcine skeletal muscle myofibrillar protein.

**Fig. 5.** Western blot of desmin after a brief exposure of myofibrils to calpain-2 and $Ca^{2+}$ followed by isolation and incubation of the myofibrils with $Ca^{2+}$. A: myofibrils were exposed to 4.2 mM free $Ca^{2+}$ and then isolated and incubated with 4.2 mM free $Ca^{2+}$ for 0 and 2 h. B: myofibrils were exposed to calpain-2 and 0, 0.1, 0.4, 0.9, 2.4 and 4.2 mM free $Ca^{2+}$, isolated and incubated with specified $Ca^{2+}$ for 2 h. Each lane was loaded with 8 μg porcine skeletal muscle myofibrillar protein.

**Fig. 6.** The effect of washing on the stability of the myofibril-bound calpain-2. After a brief exposure of myofibrils to calpain-2 and $Ca^{2+}$ to induce binding of calpain-2 to myofibrils, the myofibrils were washed for 1, 2 and 4 times, and then incubated with 5 mM $Ca^{2+}$ for 0 and 2 h. Each lane was loaded with 8 μg porcine skeletal muscle myofibrillar protein.

Fig. 1 new

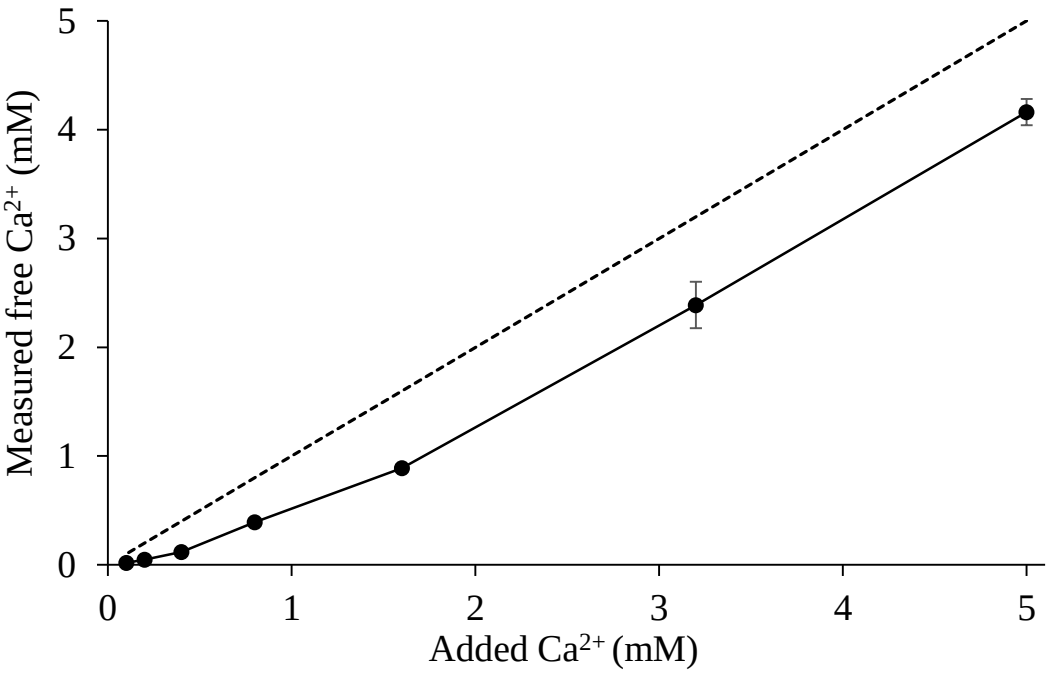


Fig. 2

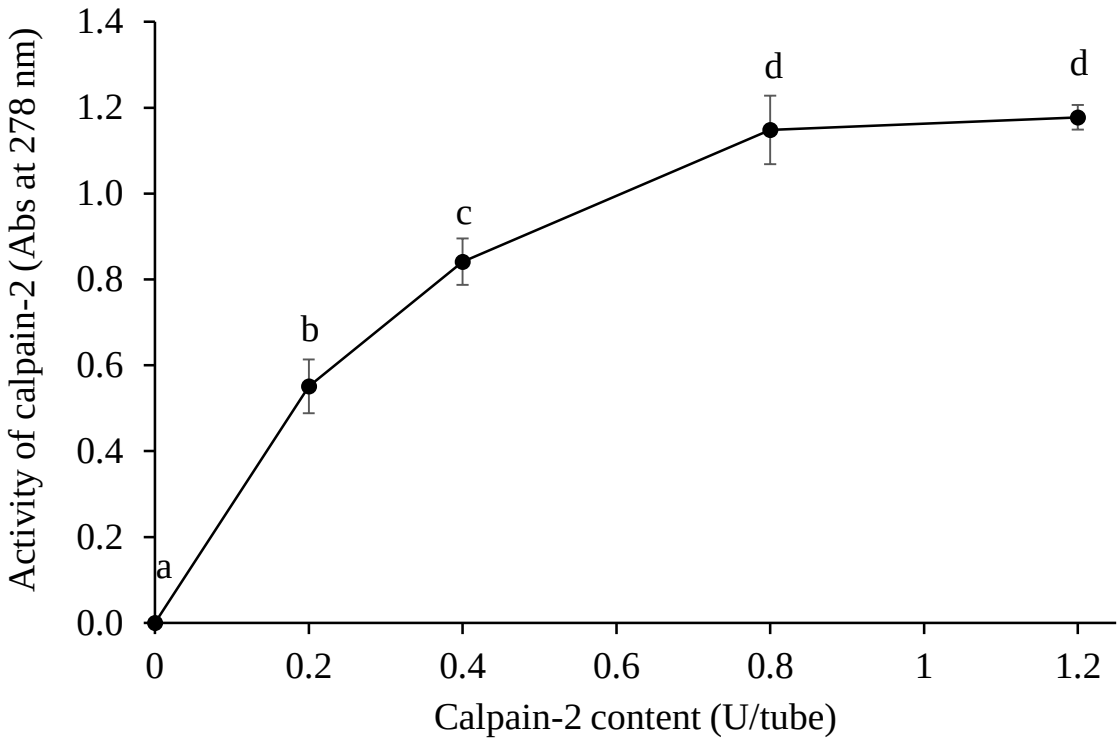

Activity of calpain-2 (Abs at 278 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
d
0
0.2
0.4
0.6
0.8
1
1.2
Calpain-2 content (U/tube)

Fig. 3

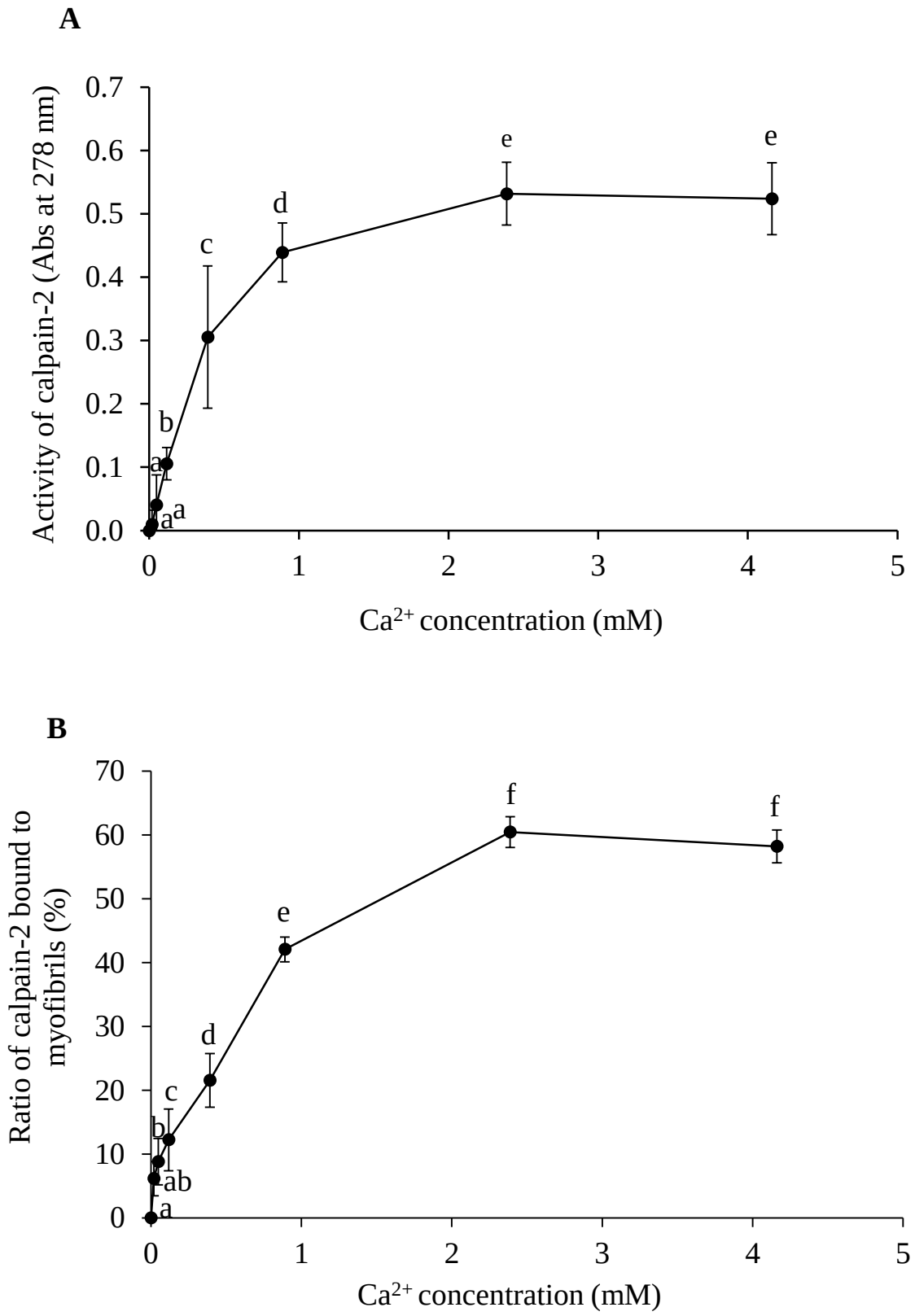

A
Activity of calpain-2 (Abs at 278 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
b
c
d
e
e
0
1
2
3
4
5
Ca2+ concentration (mM)
B
Ratio of calpain-2 bound to myofibrils (%)
70
60
50
40
30
20
10
0
a
ab
b
c
d
e
f
f
0
1
2
3
4
5
Ca2+ concentration (mM)

Fig. 4

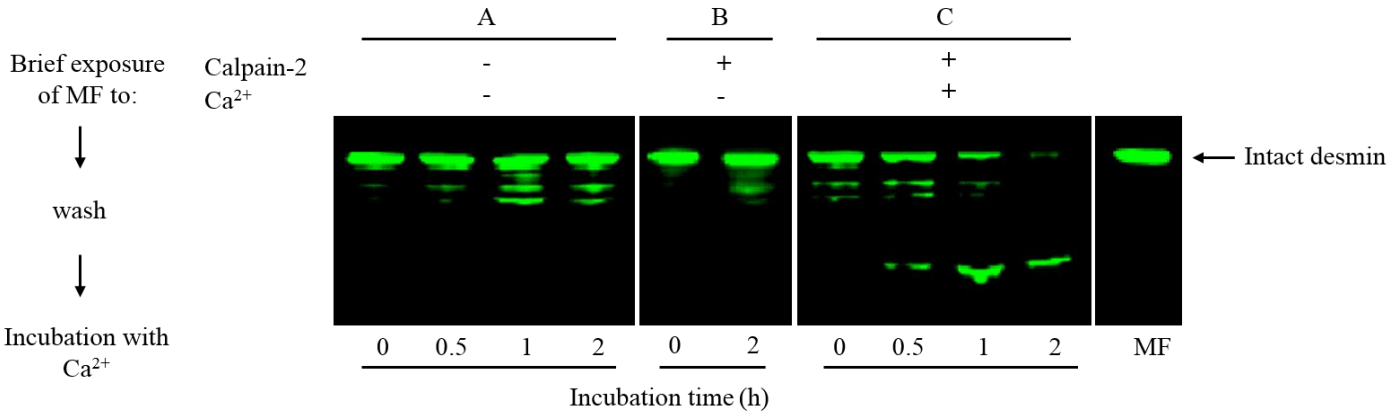

A
B
C
Brief exposure of MF to:
Calpain-2
Ca2+
-
+
+
-
-
+
wash
Incubation with Ca2+
Intact desmin
0
0.5
1
2
0
2
0
0.5
1
2
MF
Incubation time (h)

Fig. 5

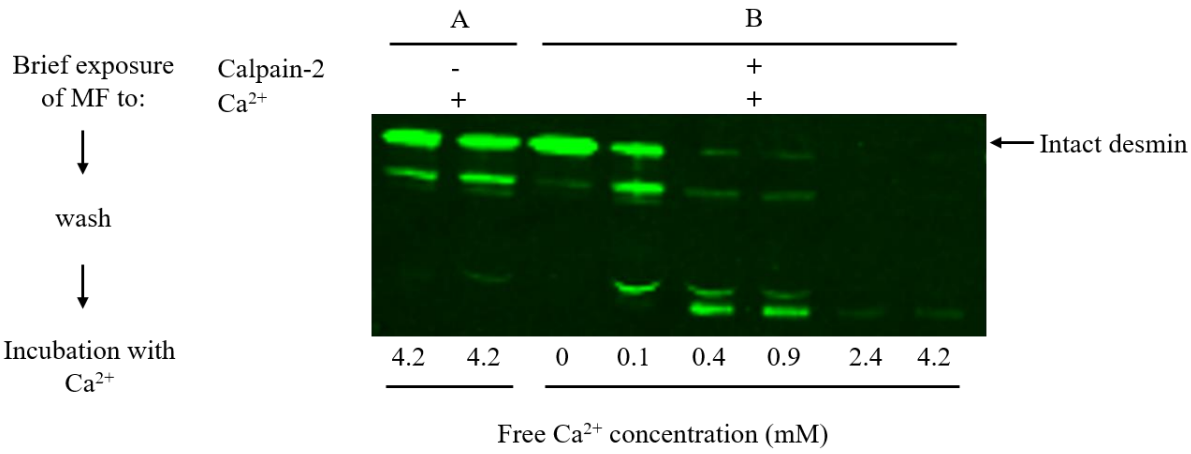
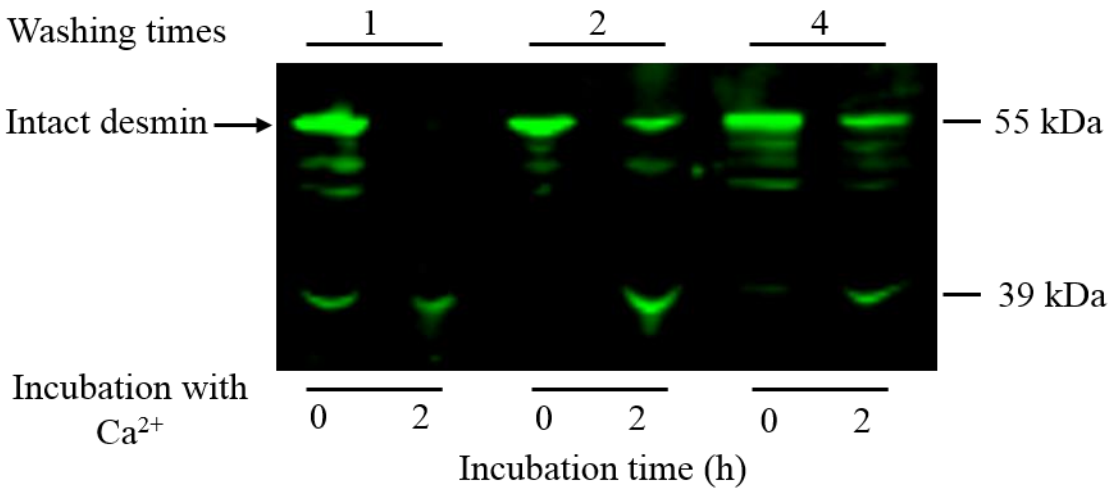

Brief exposure of MF to:
wash
Incubation with Ca2+
Calpain-2
Ca2+
A
B
-
+
+
+
Intact desmin
4.2
4.2
0
0.1
0.4
0.9
2.4
4.2
Free Ca2+ concentration (mM)

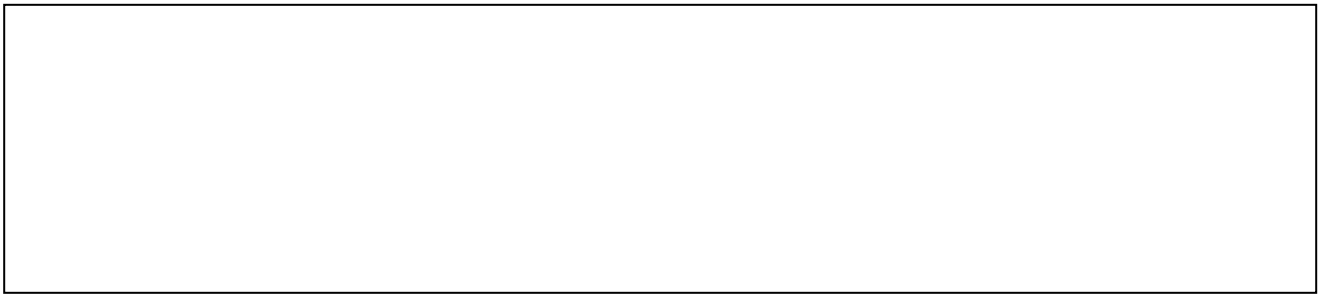
Fig. 6


Washing times
1
2
4
Intact desmin
55 kDa
39 kDa
Incubation with
Ca2+
0
2
0
2
0
2
Incubation time (h)

declaration

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



# Authorship statement

**Manuscript title**: "$Ca^{2+}$-induced binding of calpain-2 to myofibrils: New insights on myofibrillar protein degradation"

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**Analysis and/or interpretation of data**: Jian Lyu, Per Ertbjerg

**Drafting the manuscript**: Jian Lyu, Per Ertbjerg

**Revising the manuscript critically for important intellectual content**: Jian Lyu, Per Ertbjerg

**Approval of the version of the manuscript to be published**: Jian Lyu, Per Ertbjerg