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Relationship between pre-rigor temperature of pork *longissimus* muscle, myofibril-bound calpain activity and protein degradation

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ABSTRACT

The effect of pre-rigor temperature incubation on the activity and distribution in sarcoplasmic and myofibrillar fractions of calpains, and meat quality attributes was investigated. Porcine *longissimus thoracis* muscles were incubated pre-rigor at 14, 22, 30 and 38 °C to 6 h postmortem, followed by another 2 h incubation at 14 °C. Thereafter, muscles were stored at 2 °C for 1 or 4 days. With higher pre-rigor temperature, sarcoplasmic Ca^{2+} concentration, purge loss and myofibril-bound calpain-1 content increased, while shear force declined. Water-holding capacity of isolated myofibrils was lower after pre-rigor incubation at 38 °C. Desmin and troponin T degradation, and myofibril fragmentation was greater upon incubation of isolated myofibrils with added Ca^{2+} in the order 800 μM Ca^{2+} > 40 μM Ca^{2+} > no Ca^{2+}, suggesting that calpain-1 and calpain-2 were associated to myofibrils and proteolytically active with sufficient Ca^{2+}. Activity of myofibril-bound calpain-1 in muscle incubated pre-rigor at 22 and 30 °C were higher than when incubated at 14 and 38 °C. These results indicate that calpains translocate from the sarcoplasm onto myofibrils with higher pre-rigor temperature to 30 °C and the proteolytic potential of myofibril-associated calpains is thereby increased.

1. Introduction

Tenderness of meat is an important eating quality attribute which can be affected by the conditions during pre-rigor processing (Liu et al., 2021; Savell, Mueller, & Baird, 2005). The carcass chilling rate is an important factor to consider during handling after slaughter as the temperature affects the speed of pH drop of pre-rigor muscles (Kim, Luc, & Rosenvold, 2013; Savell et al., 2005). Slow cooling exposes muscle to relatively high temperature (20 to 35 °C) before rigor and accelerates the aging process during storage (Devine, Wahlgren, & Tornberg, 1999; Tornberg, 1996). However, too high (37–38 °C) pre-rigor temperature coupled with a fast pH decline may lead to tough meat (Kim, Stuart, Nygaard, & Rosenvold, 2012; Thomson, Gardner, Simmons, & Thompson, 2008; Warner, Kerr, Kim, & Geesink, 2014). The reported toughness of meat caused by high (35–40 °C) pre-rigor temperature may be due to heat-induced sarcomere shortening or reduced postmortem proteolysis due to less calpain activity (Kim, Warner, & Rosenvold, 2014).

The calpain system is believed to be a major contributor to proteolysis which is leading to increased meat tenderness during storage (Huff Lonergan, Zhang, & Lonergan, 2010). The proteolytic degradation of myofibrils leading to tenderization occurs in a muscle-specific manner (Ma & Kim, 2020) and various processing technologies are known to influence the activity of calpains (Kaur et al., 2021). Calpain-1 properties and activity are influenced by the muscle temperature postmortem (Du et al., 2017; Mohrhauser, Lonergan, Huff-Lonergan, Underwood, & Weaver, 2014; Yan et al., 2022). A relatively high pre-rigor temperature in the range of 20 to 35 °C can activate calpain-1 early postmortem and lead to rapid tenderization (Dransfeld, 1994). However, high temperature (35–40 °C) can also cause protein denaturation, calpain inactivation and thereby limit calpain activity and degradation of myofibrillar proteins, finally resulting heat-induced toughening (Kim et al., 2014). Decrease in extractable activity of calpains measured by a method such as casein zymography has been regarded as an indicator of the degree of their involvement in postmortem degradation of myofibrillar proteins. A faster decrease in activity of sarcoplasmic calpains has often been observed in muscle upon high (33–40 °C) temperature incubation (Hwang & Thompson, 2001; Liu, Ruusunen, Puolanne, & Ertbjerg, 2014) and at longer incubation times in the range of 25 to 30 °C (Pomponio & Ertbjerg, 2012). The decline in sarcoplasmic calpain activity were taken as an indication early postmortem activation. It has been suggested that calpains first bind to myofibrils, and then degrade structural proteins during storage while being bound to myofibrils (Lyu

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& Ertbjerg, 2021). The level of calpain-1 being associated to myofibrils has been reported to increase gradually postmortem (Boehm, Kendall, Thompson, & Goll, 1998; Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). In pork the level of myofibril-bound calpain-1 reached a maximum at day 6 postmortem (Lyu & Ertbjerg, 2022). However, the exact cause for the decrease in extractable activity of calpains after high (35–40 °C) pre-rigor temperature is not known. Possible reasons may be limited calpain activity in protein denaturing conditions caused by high pre-rigor temperature and early activated calpains becoming bound to myofibrils. Information about how the temperature influences the proteolytic potential and level of association of calpains to myofibrils, as well as its correlation with the meat tenderness is lacking. Therefore, we studied the influence of pre-rigor temperature on the translocation and proteolytic activity of calpains, and their relationship to meat quality in pork muscle.

2. Material and methods

2.1. Sample preparation

Five *longissimus thoracis* (LT) muscles were obtained from five different pigs (from the cross Landrace × Yorkshire × Duroc and carcass average weight of 89 Kg) at a local slaughterhouse (HKScan Slaughterhouse, Forssa, Finland). After the pigs had been CO_2 stunned and slaughtered, then the loins were cut off around 1 h post-mortem. After measurement of initial pH and temperature at 1 h, each loin was rapidly separated into four slices and each slice was further divided into two parts (about 11 × 6 × 3 cm along the length of muscle). The muscle slices were weighed, sealed in plastic bags, and then water-bath incubated at 14, 22, 30 and 38 °C from approximately 1.5 h postmortem until 6 h postmortem, and thereafter incubated for additional 2 h at 14 °C before being cold-stored for 1 or 4 days at 2 ± 1 °C. The water-bath incubation was initiated during the transport to the laboratory at University of Helsinki. The specific temperature of water bath was kept constant and regulated by hot water and ice.

2.2. Measurements of temperature, color and pH

Measurements of initial temperature and pH were done 1 h postmortem (before cutting and incubation), and thereafter pH measurements were conducted at 2, 4, 6, 8 and 30 h postmortem. The pH of meat homogenates was determined by using a pH electrode (Mettler-Toleda Inlab 427). Aliquots of muscle samples (0.4 to 0.5 g) were homogenized with 5 mL of 5 mM sodium iodoacetate and 150 mM KCl (Jeacocke, 1977). The center temperature of the large slices (section 2.1) was measured and the initial temperature of pork was 37.7 ± 0.8 °C. The center temperature of muscles incubated at 22, 30 and 38 °C were at 2 h postmortem within one degree of the temperature of the water bath, respectively, while the center temperature of muscles in the 14 °C water bath was 16 ± 1 °C. Average values of (L*, a* and b*) after pre-rigor incubation at 14, 22, 30 and 38 °C were (53.65 ± 1.87, 6.58 ± 1.00 and 5.00 ± 1.50), (50.18 ± 1.87, 7.47 ± 1.03 and 4.50 ± 1.23), (53.14 ± 3.76, 6.40 ± 1.58 and 4.23 ± 1.02) and (60.81 ± 3.16, 8.18 ± 1.59 and 7.53 ± 1.50), respectively,

2.3. Exudate loss and shear force

The indicators of exudate loss and the shear force were determined according to the methods by Zhang and Ertbjerg (2018) with minor modifications. For accumulated purge loss measurement, after pre-rigor incubation and cold storage, the bag was opened, then the surface liquid of the meat was gently removed with filter paper. The purge loss (%) was calculated by measuring the initial weight and subtracting the weight after storage. The meat was vacuum packaged after measurement of the day-4 purge loss then cooked 1 h in a water bath (72 °C) and after heat treatment cooled for 30 min at 0 °C. Thereafter, cooking loss (%) was calculated as the weight loss during cooking. The initial weight subtracted the weight after cooking was regarded as total loss (%). Allo-Kramer shear force was determined according to the method described by Liu et al. (2014). The cooked meat was sliced into 8–10 pieces with the size of 20 × 20 × 6 mm (fiber axis along 20 mm direction). The weight (g) of muscle pieces was recorded and then cut across the fiber axis by Instron Model 6625 (Instron Co, Canton, MA). The maximum load (N) for each muscle piece was recorded and then corrected by the sample weight. The unit of shear force was shown as N/g.

2.4. Measurement of free calcium

Sarcoplasmic Ca^{2+} level was determined as outlined by Hopkins and Thompson (2001) using the method of Ca^{2+} standard addition as described by Pomponio and Ertbjerg (2012). Fresh meat (15 g) was finely diced and 2 mL of supernatant was collected after 30 min of centrifugation at 20,000 ×*g*, 4 °C. The level of free Ca^{2+} in the supernatant was measured by using a selective electrode (Mettler-Toledo perfectION™ Combination Electrode, Greifensee, Switzerland) for Ca^{2+} after addition of 40 μL of 4 M KCl. All the samples were determined by the method of Ca^{2+} standard addition.

2.5. Activity of calpains associated to myofibrils

Myofibril-bound calpain activity was determined according to the method reported by Lyu and Ertbjerg (2022). The Ca^{2+} induced proteolytic activity of myofibril-bound calpains was assessed by adding 40 and 800 μM Ca^{2+}. The lower concentration of 40 μM was added for activation of myofibril-bound calpain-1, whereas 800 μM Ca^{2+} for activation of both myofibril-bound calpain-1 and calpain-2. Three independent meat batches were prepared for this assay, and aliquots of all five LT muscles were pooled in each batch. Meat samples (1.3 g) of every animal were collected and diced followed by addition of rigor buffer (20 mM Tris-HCl, 2 mM EGTA, 75 mM KCl, 2 mM $MgCl_2$, pH 7.0) at 4 °C at a ratio of 1: 4 (w: v). The myofibril pellet was obtained after 3 × 12 s of homogenization at 13,500 rpm and 10 min of centrifugation at 10,000 ×*g*, 4 °C. The myofibril pellet was re-suspended in washing buffer (20 mM Tris-HCl, 75 mM KCl, 2 mM $MgCl_2$, pH 7.0) and centrifuged 5 min at 10,000 ×*g*, 4 °C. A 15 mg protein/mL suspension was obtained by dispersing the myofibril pellet in incubation buffer (100 mM Tris-HCl, 75 mM KCl, 2 mM $MgCl_2$, pH 7.0). $CaCl_2$ solution (50 mM $CaCl_2$, 75 mM KCl, 100 mM Tris, 2 mM $MgCl_2$, pH 7.0) was mixed with 2 mL myofibril suspension to the final Ca^{2+} concentration (either 40 μM or 800 μM). After 2 h incubation at 25 °C, 150 μL EGTA solution (20 mM EGTA, 75 mM KCl, 100 mM Tris, 2 mM $MgCl_2$, pH 7.0) was added to stop the reaction. All reaction tubes were placed in an ice container and processed for water-holding capacity (WHC) of myofibrils, particle size and western blot (desmin and troponin-T) analysis. For control treatments, the same volume of incubation buffer was added to replace $CaCl_2$. The purpose of the control was to indicate the proteolysis caused by other peptidases (myofibrils were incubated without Ca^{2+}) than myofibril-bound calpains (myofibrils were incubated with Ca^{2+}) during the 2 h-incubation. The level of intact desmin after 2 h incubation of isolated myofibrils without Ca^{2+} (0 μM Ca^{2+}) was always around 10% less than that without incubation (Lyu & Ertbjerg, 2022), and the control can thus indirectly reflect the postmortem proteolysis during aging.

2.6. Particle size

The distribution of particle size of the myofibril suspension was detected by Mastersizer 3000, a laser diffraction particle size analyser (Malvern Instruments Ltd., Malvern, UK). Each myofibril suspension was dispersed in distilled water and analyzed five times. The myofibril particle was taken as non-spherical, and the absorption coefficient and refractive index was set to 0.01 and 1.46, respectively. The surface-

weighted mean diameter D(3,2) was taken as the mean particle size of the suspension.

2.7. WHC of myofibrils

WHC of the myofibril pellet was measured as described by Zhang and Ertbjerg (2018). After incubation, each myofibril suspension was centrifuged (10 min, 20,000 ×*g*, 25 °C). The myofibril pellet was suspended in 1.7 mL cold MES buffer (75 mM KCl, 100 mM MES, 2 mM $MgCl_2$, pH 5.4) and centrifuged for 5 min at 2400 ×*g*. The myofibril pellet was then dried at 100 °C overnight. The weight of the pellet before and after drying was used to calculate the WHC of myofibrils. Correction for the weight of the dry myofibrillar protein was done by subtracting residual salt from the MES buffer. Water content retained by 1 g of protein was taken as the WHC of myofibrils.

2.8. SDS-PAGE and western blot

Myofibrils and supernatant preparation for determination of calpain-1, and gel electrophoresis and western blots were conducted according to Lyu and Ertbjerg (2022). The pooled supernatants from all pork loins (1 h post-mortem) was taken as a reference standard for quantification. The transfer procedure was conducted at 30 V overnight for calpains, and 1 h for desmin and troponin T. Calpain-1, troponin T and desmin were detected by MA3–940 (Invitrogen, Carlsbad, CA), T6277 (Sigma, St. Louis, MO, USA) and D8281 (Sigma) at 1:1000, 1:15,000 and 1:3000, respectively. The membranes were then probed with IRDye® 800 CW Donkey anti-mouse for calpain-1 and troponin T, and IRDye® 800 CW Donkey anti-rabbit for desmin, respectively.

Fluorescence intensity of band for desmin, troponin T and calpain-1 (native and autolyzed calpain-1 altogether) was quantified by Odyssey Infrared Imaging System-CLx (LI-Cor Cop, Lincoln, NE). The integrated intensity of intact desmin divided by the average of the 1 h reference standard on each membrane was taken as the relative intensity for desmin (%). The degradation of troponin T (%) was expressed by intensity of troponin T degradation products divided by the average of its 1 h standard. For quantification of sarcoplasmic calpains the density of bands migrating at 80 kDa, 78 kDa and 76 kDa were combined. The standard loaded with the same protein content as other samples was considered as 100%; while 25% of the standard (relative to sarcoplasmic samples) was loaded for the myofibril-bound calpains because of the smaller calpain-1 amount in the myofibrillar fraction. The myofibril-bound calpain-1 content was corrected to be comparable to the muscle content of sarcoplasm calpain-1 assuming a 2: 1 ratio of myofibrillar: sarcoplasmic protein.

2.9. Statistical analysis

Duplicates for every pork loin were done for quantification of calpain-1 by western blot. For each meat batch, 4 replicates were performed for desmin and troponin T; 5 replicates were performed for particle size and five measurements were done for each repeat; 12 replicates were conducted for each of the incubation suspensions for WHC. The average of pH with time post mortem for each pre-rigor incubation temperature was modeled as cubic splines.

Purge loss, sarcoplasmic Ca^{2+}, relative intensity of bands (sarcoplasmic and myofibril-bound calpain-1, intact desmin, degraded troponin-T) were analyzed by general linear model (Univariate). Muscle number was set as random effect, pre-rigor incubation temperature and storage time were set as fixed effects with an interaction term for temperature × time for purge loss, sarcoplasmic Ca^{2+}, relative intensity of bands of sarcoplasmic and myofibril-bound calpain-1. For band intensity of desmin and troponin-T, pre-rigor incubation temperature and added Ca^{2+} concentration were used as fixed effects with an interaction term for temperature × Ca^{2+} concentration. Cook loss, total loss, shear force, mean particle size and WHC were analyzed by using one-way ANOVA, and pre-rigor temperature was set as fixed effect. Mean values and standard errors were generated from the models. IBM SPSS Statistics 25 software was used for the data analysis. The significant differences between mean values were evaluated by Bonferroni test at a level at $P < 0.05$.

3. Results

3.1. Temperature and pH

The pH dropped faster when the incubation temperature increased (Fig. 1). Muscles incubated pre-rigor at 38 °C exhibited the fastest decline of pH and at 6 h postmortem it reached the final pH. The final pH for all muscles was around 5.6.

3.2. Exudate loss

The incubation temperature showed a pronounced influence on the purge loss and cook loss ($P < 0.01$) (Tables 1 and 2), and the purge loss increased ($P < 0.01$) throughout the storage period. Muscle samples incubated pre-rigor at 38 °C exhibited PSE-like characteristics with greater ($P < 0.05$) L*-values (section 2.2) and purge loss but pronouncedly lower ($P < 0.05$) cook loss than at the other lower temperatures (Table 2). Total loss was unaffected by the temperature incubation (Tables 1 and 2).

3.3. Allo-Kramer shear force

The shear force value on day 4 was greatly influenced by the pre-rigor temperature ($P < 0.01$, Table 1). The shear force of meat incubated pre-rigor at 38 °C was lower than meat with 14, 22 and 30 °C incubation ($P < 0.05$). No difference was observed between the shear force of meat incubated at 14 and 22 °C, but it was greater ($P < 0.05$) compared with that of meat at 30 °C (Table 2).

3.4. Sarcoplasmic Ca^{2+}

The sarcoplasmic Ca^{2+} concentration was affected ($P < 0.01$) by both the storage time and pre-rigor incubation temperature (Table 1). The sarcoplasmic Ca^{2+} level for the muscles became greater with storage time (Table 2). Muscles incubated at 38 °C showed a faster Ca^{2+} increase than the other temperatures and increased from 230 μM at 8 h to 360 μM at day 4 postmortem ($P < 0.05$). The free Ca^{2+} concentration in muscles

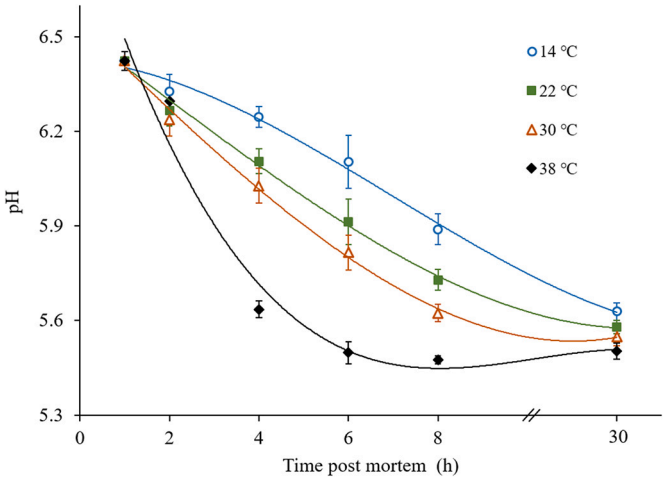

Fig. 1. Postmortem pH decline of porcine *longissimus thoracis* muscles. The lines were fitted as cubic splines. The muscles were incubated at 14, 22, 30 and 38 °C to 6 h postmortem and thereafter incubated at 14 °C for another 2 h and were then stored at 2 °C for 1 or 4 days. Mean values with standard errors are shown.

Table 1
Effect of storage, pre-rigor temperature incubation and their interaction on free Ca^{2+}, purge loss, cook loss, total loss, shear force, content of sarcoplasmic and myofibril-bound calpain-1 (A); and effect of storage, pre-rigor temperature incubation, incubation of isolated myofibrils with added Ca^{2+}, and their interaction on water holding capacity (WHC) of myofibrils, particle size and degradation of desmin and troponin T (B).

Effects (A)	Free Ca^{2+}	Purge loss	Cook loss	Total loss	Shear force	Sarcoplasmic calpain-1	Myofibril-bound calpain-1
Temperature	**	**	**	NS	**	**	**
Storage	**	**	–	–	–	**	NS
Temperature × Storage	*	NS	–	–	–	NS	NS

Effects (B)	WHC	Desmin degradation	Troponin T degradation	Particle size
Temperature	**	**	**	**
Storage	**	**	**	*
Added Ca^{2+}	**	**	**	**
Temperature × Storage × added Ca^{2+}	**	**	**	**

NS: not significant.
* $P < 0.05$.
** $P < 0.01$.

Table 2
Exudate loss, concentration of free Ca^{2+} and shear force of porcine muscles after pre-rigor temperature incubation during storage.

		Temperature (°C)				SEM
		14	22	30	38	
Accumulated purge loss (%)	24 h	1.2^{aw}	1.9^{bw}	2.0^{bw}	8.2^{cw}	0.4
	48 h	3.5^{ax}	4.2^{bx}	4.6^{bx}	10.4^{cx}	
	72 h	5.3^{ay}	5.6^{by}	6.3^{by}	11.4^{cy}	
	96 h	6.9^{az}	7.9^{bz}	7.7^{bz}	12.7^{cz}	
Cook loss (%)	96 h	31.0^{a}	30.3^{a}	29.8^{a}	26.6^{b}	0.4
Total loss (%)	96 h	35.7^{a}	35.8^{a}	35.2^{a}	35.4^{a}	0.4
Ca^{2+} concentration (μM)	8 h	50^{aw}	66^{bw}	92^{bw}	234^{cw}	13.9
	24 h	60^{aw}	129^{bw}	138^{bw}	266^{cw}	
	96 h	166^{ax}	316^{bx}	344^{bx}	358^{cx}	
Allo-Kramer shear force (N/g)	96 h	146^{a}	144^{a}	135^{b}	110^{c}	2.0

SEM: Standard error of the mean.
$^{a–c}$Within each row, mean values with the same letter do not differ ($P > 0.05$).
$^{w–z}$Within each column, mean values with the same letter do not differ ($P > 0.05$).

incubated at 14 °C increased gradually and it was lower than that of 22 and 30 °C ($P < 0.05$).

3.5. Sarcoplasmic and myofibril-bound calpain-1

Pre-rigor incubation affected ($P < 0.01$) the amount of calpain-1 in the sarcoplasm and myofibrils (Fig. 2 and Table 1). Muscles incubated at 14 and 22 °C showed more calpain-1 in the sarcoplasm than those at 30 and 38 °C at both day 1 and day 4 ($P < 0.05$). However, the myofibril-bound calpain-1 content increased with elevated temperature and it was more following pre-rigor incubations at 30 and 38 °C compared to 14 °C ($P < 0.05$). Storage affected ($P < 0.01$) the content of sarcoplasmic calpain-1 but not myofibril-bound calpain-1. In meat incubated pre-rigor at 14, 22 and 30 °C, the amount of sarcoplasmic calpain-1 on day 4 was much higher than that of day 1. However, the myofibril-bound calpain-1 content slightly decreased on day 4. This might be caused by an effect of elevated pre-rigor temperature to accelerate translocation of calpain-1 from sarcoplasm to myofibrils. This suggests that the myofibril-bound calpain-1 content reached its maximal before day 4 and subsequently myofibril-bound calpain-1 started to be released back into the sarcoplasm. However, in meat incubated at 38 °C, the amount of sarcoplasmic and myofibril-bound calpain-1 changed only little during 4-day storage.

3.6. Degradation of desmin and troponin T

The myofibril-bound protease activity was shown by monitoring desmin and troponin T degradation after incubation of isolated myofibrils with Ca^{2+} (Fig. 3). Pre-rigor temperature incubation and storage affected ($P < 0.01$) desmin degradation (Table 1). After incubation with Ca^{2+}, desmin in myofibrils was degraded in the order 800 μM > 40 μM > 0 μM ($P < 0.05$). For both of the storage days, the content of intact desmin in muscle incubated at 14 °C was greater ($P < 0.05$) than those incubated at 22 and 30 °C, and less ($P < 0.05$) than those incubated at 38 °C. On day 1, a similar amount of intact desmin in meat at 14 and 38 °C was found after incubation of myofibrils without Ca^{2+}. After myofibrils were incubated with 40 μM Ca^{2+}, more degradation of desmin was found in meat at 14 °C than at 38 °C. However, after myofibrils were incubated with 800 μM Ca^{2+}, much more degradation of desmin was observed in meat incubated pre-rigor at 38 °C than at 14 °C. Troponin T was more degraded in muscles at 14, 22 and 30 °C than in those at 38 °C after isolated myofibrils were incubated with 0 and 40 μM Ca^{2+}. After incubation of isolated myofibrils with 800 μM Ca^{2+}, troponin T degradation products increased ($P < 0.05$) in muscles at all temperatures and a greater increase was observed in muscles incubated pre-rigor at 38 °C than at the lower temperatures.

3.7. WHC of myofibrils

The WHC of myofibrils in muscles kept at 38 °C during pre-rigor incubation was lower ($P < 0.05$) on day 1 and day 4 than those kept at lower temperatures (Tables 1 and 3). After myofibrils were incubated with 800 μM Ca^{2+}, the WHC of myofibrils on day 1 increased pronouncedly ($P < 0.05$) at all temperatures compared to incubations with 0 and 40 μM Ca^{2+}.

3.8. Particle size

Pre-rigor temperature incubation affected the particle size of myofibrils ($P < 0.01$) (Tables 1 and 3). The mean particle size decreased with storage time, and at day 4 the control myofibrils (incubated without Ca^{2+}) of muscles incubated pre-rigor at 30 °C were more fragmented as the particle size was smaller. In muscle incubated at 14, 22 and 30 °C, the particle size of day 1 myofibrils decreased upon incubation with Ca^{2+} in the order 800 μM > 40 μM > 0 μM ($P < 0.05$), except the muscle at 38 °C. At that temperature 40 μM incubation did not affect the particle size compared to those without Ca^{2+}, and these parameters only decreased ($P < 0.05$) when incubation of isolated myofibrils were done with 800 μM Ca^{2+} in comparison with 0 and 40 μM Ca^{2+}.

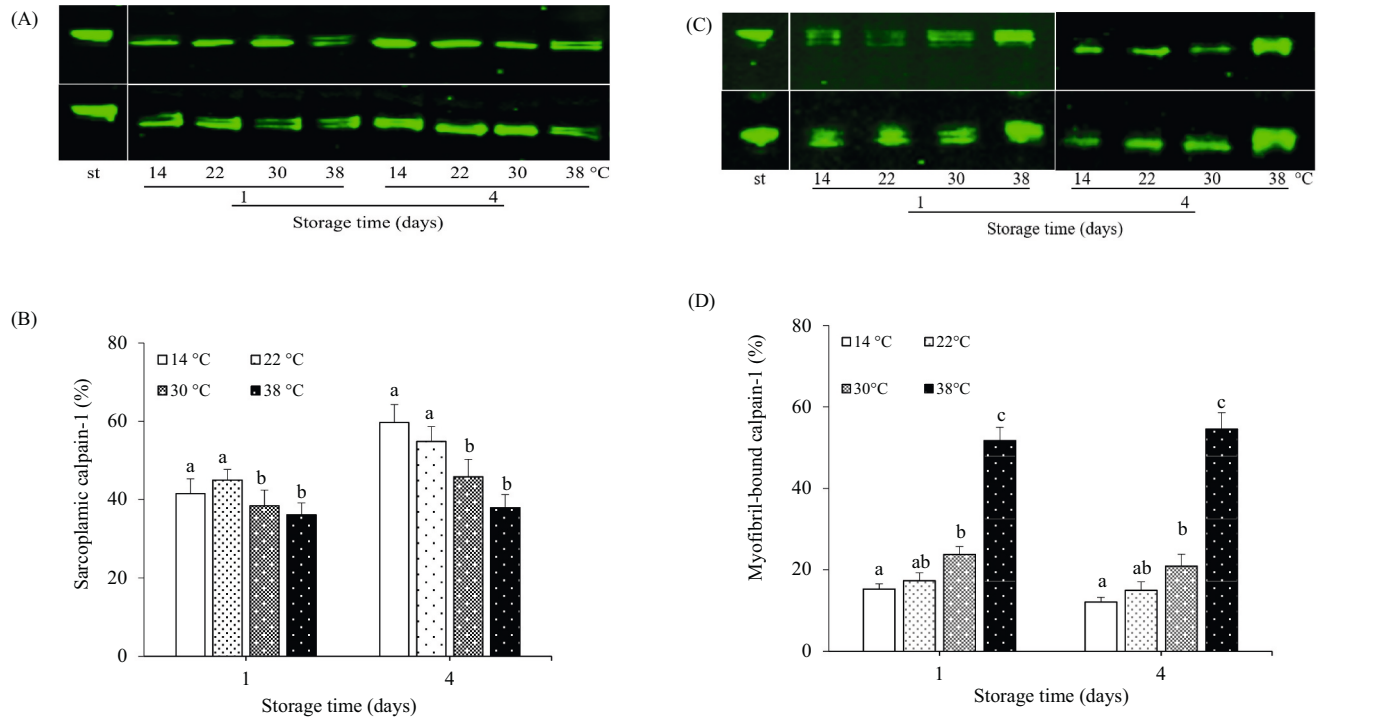

Fig. 2. Western blot for calpain-1 in the sarcoplasm (A) and myofibril (C) fractions of porcine *longissimus thoracis* muscle and relative intensity of calpain-1 bands in the sarcoplasm (B) and myofibrils (D). The pooled supernatant extracted from all 1-h pork loins (taken as 100%) was used as the standard reference (st). Each lane was loaded with 15 μg of protein except 3.7 μg of protein was loaded onto the st lanes for myofibrils. [a-c]Within traits and storage time, means with the same letter do not differ ($P > 0.05$).

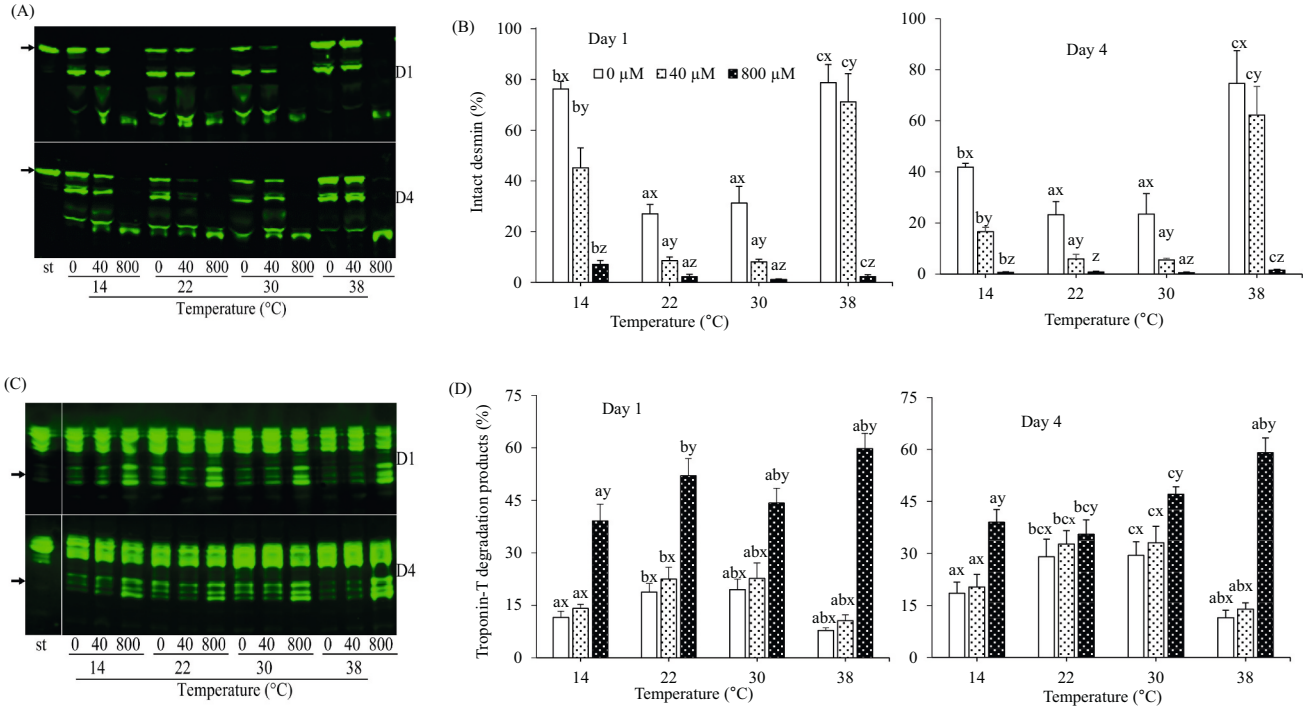

Fig. 3. Western blot for desmin (A) and troponin T (C) and relative intensity for desmin (B) and troponin T (D) bands after incubation of myofibrils at day 1 or day 4 with 0, 40 and 800 μM of Ca^{2+}. Standard lanes (st) were the pooled myofibrillar protein extracted from all 1-h pork loins (taken as 100%). Each lane was loaded with 10 μg of myofibrillar protein. [a-c, x-z]Within temperature (a-c) and Ca^{2+} concentrations (x-z), means with the same letter do not differ ($P > 0.05$). Arrows in (A) and (C) indicate intact desmin and degradation products of troponin T, respectively.

Table 3
Water-holding capacity (WHC) and mean particle size of myofibrils after incubation with different concentrations of Ca^{2+} (0, 40 and 800 μM).

		Temperature (°C)								
		Day 1				Day 4				
	Ca^{2+} (μM)	14	22	30	38	14	22	30	38	SEM
WHC (g H_2O/g protein)	0	9.14^{cd}	9.07^{c}	9.39^{cde}	8.50^{b}	9.13^{cd}	9.47^{def}	9.36^{cde}	7.98^{a}	0.03
	40	9.07^{c}	9.19^{cd}	9.34^{cde}	8.25^{ab}	9.11^{cd}	9.66^{efg}	9.26^{cd}	8.05^{a}	
	800	10.14^{i}	10.10^{hi}	10.18^{i}	9.12^{cd}	9.77^{fgh}	9.87^{ghi}	9.39^{cde}	8.31^{ab}	
Mean particle size (μm)	0	114^{a}	107^{b}	96^{cd}	96^{cd}	91^{d}	68^{f}	57^{gh}	76^{e}	
	40	64^{fg}	66^{f}	77^{e}	100^{bc}	67^{f}	56^{h}	53^{h}	76^{e}	2.0
	800	28^{ij}	34^{i}	34^{i}	34^{i}	25^{j}	27^{ij}	31^{ig}	28^{ij}	

SEM: Standard error of the mean.
$^{a–j}$Within traits, mean values with the same letter do not differ ($P > 0.05$).

4. Discussion

The distribution of the size of particles can estimate the degree of myofibrillar fragmentation that occurs during postmortem proteolysis (Lametsch, Knudsen, Ertbjerg, Oksbjerg, & Therkildsen, 2007). Desmin is a structural and troponin T a regulatory myofibrillar protein and their degradation is an indicator for proteolysis during aging (Huff-Lonergan et al., 1996; Yan et al., 2022). In this study, the association of calpain-1 to myofibrils (Fig. 2) together with degradation of myofibrillar proteins (Fig. 3) and reduced particle size (Table 3) in the incubation assays with different Ca^{2+} concentrations suggest that calpains bind to myofibrils. Furthermore, if the Ca^{2+} level is sufficient then they are able to degrade myofibrillar proteins during storage. Proteolysis in muscles incubated pre-rigor at 22 and 30 °C was greater than when incubated at 14 °C and 38 °C as observed by more degraded desmin and troponin T and greater fragmentation of myofibrils. Additionally, desmin was more degraded in meat incubated pre-rigor at 22 and 30 °C, than in that incubated at 14 °C, when myofibrils subsequently were incubated with 40 and 800 μM Ca^{2+} (Fig. 3A and B). This suggests that more calpains bind to myofibrils in higher temperature incubations, which coincides with the quantification result of myofibril-bound calpain-1 (Fig. 2D). These results are presumably linked to the greater sarcoplasmic Ca^{2+} concentration in the muscles incubated pre-rigor at 22 and 30 °C compared to those incubated at 14 °C (Table 2). Decreased pH and increased temperature can lead to an increased level of sarcoplasmic Ca^{2+} due to reduced stability of mitochondria and sarcoplasmic reticulum under this condition (Whiting, 1980). In agreement with the current results, it has been reported that more purified calpain-2 bound to isolated myofibrils after a suspension of isolated myofibrils and purified calpain-2 was briefly exposed to the increased Ca^{2+} (Lyu & Ertbjerg, 2021). Similarly, increased Ca^{2+} concentration with elevated incubation temperature followed by faster calpain-1 autolysis and protein degradation have previously been observed in beef (Hwang, Park, Cho, & Lee, 2004). It can thus be hypothesized that with increased temperature, calpain translocation from sarcoplasm to myofibrils and its proteolytic activity become greater due to the higher level of sarcoplasmic Ca^{2+}, ultimately leading to increased meat tenderization. Similarly, earlier activation of calpain-1 and calpain-2 and greater extent of myofibrillar fragmentation degree were observed in porcine muscle incubated at 25 and 30 °C compared to that of 2 and 15 °C (Pomponio & Ertbjerg, 2012). Desmin was substantially degraded from day 1 to day 4 in the samples incubated pre-rigor at 14 °C, while at 22 and 30 °C no obvious degradation of desmin during this period was observed. This suggests that proteolysis following incubations at 14 °C occurs gradually during the storage period, while at 22 and 30 °C protein degradation is more rapid early postmortem and become relatively slower in later storage. Similarly, accelerated calpain activation early postmortem and decreased calpain activity and proteolysis at later storage were observed in porcine (Liu et al., 2014), bovine (Hwang et al., 2004) and ovine (Geesink, Bekhit, & Bickerstaffe, 2000) muscle after treatments with elevated pre-rigor temperatures.

The impact of high pre-rigor temperature on proteolysis and tenderness of meat during storage has been reported by many studies, but different results were found among them. Similar to our results, lower shear force following pre-rigor incubation of pork at 40 °C (Liu et al., 2014) and beef at 35 °C (Balan et al., 2019) was reported when compared with incubation at lower temperatures, and it was thought to be due to the early activation of calpain-1 as evidenced by reduced calpain-1 activity in casein zymography and reduced amount of native calpain-1 in western blot, respectively. In contrast, higher shear force values were found in ovine (Geesink et al., 2000) and bovine muscles (Kim et al., 2012) following high pre-rigor temperature and the toughness was explained by an opposing influence of muscle shortening on meat tenderness. In the current study, almost no proteolysis from day 1 to day 4 was observed following 38 °C pre-rigor incubation, although the free Ca^{2+} concentration was greater than incubation at lower temperatures. Myofibrils from the 38 °C incubation showed the most abundant level of bound calpain-1 (Fig. 2), however, it had apparently lost its activity as observed by the low decrease in particle size (Table 3) and the low degree of protein degradation (Fig. 3) after the myofibrils were incubated with 40 μM Ca^{2+}. In addition, unlike in lower temperature incubations on day 4 where calpain-1 autolyzed completely to the 76 kDa form, there was still some partly autolyzed 78 kDa calpain-1 in both the sarcoplasm and myofibrils following the 38 °C incubation (Fig. 2A and C). Similarly, after storage partly autolyzed calpain-1 was found in deep *semimembranosus* muscle in beef (Kim, Lonergan, & Huff-Lonergan, 2010) and beef incubated pre-rigor at 38 °C (Kim et al., 2012). All these results indicate that calpain-1 had no or only little effect on protein degradation following 38 °C incubation, and thus it seems that calpain-1 is not the reason for the low shear force in muscles incubated at 38 °C. However, a substantial amount of calpain-1 was found in the myofibrillar fraction on both day 1 and 4 and no change of its amount was observed during storage (Fig. 2C and D). The combination of low pH (< 5.8) and high temperature (35–40 °C) tends to cause pronounced protein denaturation in muscle (Honikel & Kim, 1986), including phosphorylase (Fischer, Hamm, & Honikel, 1979; Liu et al., 2014; Zhu, Ruusunen, Gusella, Zhou, & Puolanne, 2011), creatine kinase (Liu et al., 2014; Scopes, 1964) and myosin (Stabursvik, Fretheim, & Frøystein, 1984), as indicated by reduced activity and solubility or increased amount in myofibrillar fraction. Therefore, one possible explanation is that calpain-1 denatured and precipitated onto the myofibrils due to high pre-rigor temperature in combination with the fast decrease of pH. Thus, this early denatured calpain-1 could be the reason for the presence of incompletely autolyzed calpain-1 even after long postmortem storage, as it will not undergo the normal process of being activated by increased Ca^{2+} concentration, binding to the myofibrils and degrading the structural proteins. Similar to the results in the present study, after storage the presence of partly autolyzed calpain-1 (78 kDa) in deep *semimembranosus* beef muscle, which had a higher muscle temperature and faster pH decline than the superficial muscle, was suggested to be attributed to the protein-denaturing condition caused by higher pre-rigor temperature combined with faster decline of pH, and thereby

leading to inactivation of calpain-1 and limited postmortem proteolysis (Kim et al., 2010). Kim et al. (2012) reported similar results in beef pre-rigor treated at 38 °C compared with 15 °C. The sarcoplasmic Ca^{2+} concentration in muscle incubated at 38 °C increased rapidly and reached 0.36 mM on day 4 (Table 2), a level which is sufficient to initiate activation of calpain-2 (Lyu & Ertbjerg, 2022), and induce translocation of some calpain-2 from the sarcoplasm to the myofibrils. After isolated myofibrils from the muscles incubated pre-rigor at 38 °C were incubated with 800 μM Ca^{2+}, substantial troponin T and desmin degradation and decrease in particle size were observed. Additionally, the intact desmin almost disappeared, suggesting that there was sufficient myofibril-bound calpain-2 to break down myofibril proteins upon activation with Ca^{2+}. We therefore speculate that after pre-rigor incubation at 38 °C, activation of calpain-2 then induced that it became bound to myofibrils initiated by a significant increase of sarcoplasmic Ca^{2+}. Following association to myofibrils the sensitivity of calpain-2 to high temperature might be decreased and thereby preserve proteolytical potential during storage. However, the lack of myofibrillar protein degradation and myofibril fragmentation during cold storage after 38 °C pre-rigor incubation suggests that the free Ca^{2+} concentration was sufficient to induce binding to myofibrils, but not for calpain-2 to have enough activity to degrade structural proteins. Therefore, it is an open question why this temperature group showed the lowest shear force. We speculate that the texture was affected by 1) an altered rigor process influencing the rigor bond formation as the muscles entered rigor earlier and at a higher temperature, 2) by altered protein structures due to the protein denaturation such as myosin, occurring at the combination of high temperature and low pH and 3) by proteolytic tenderization during the early stage of the cooking process possibly involving calpain-2 activity. Liu et al. (2014) measured sarcomere length and found that pork incubated pre-rigor at 0 and 40 °C had shorter sarcomeres, and they thereby suggested that heat shortening occurred to the muscles incubated pre-rigor at 40 °C. Ertbjerg and Puolanne (2017) reviewed that fracture of super-contracted sarcomeres may explain the decreased toughness of muscles when the shortening is >40%. Super-contraction induced by heat shortening could thus be one of the reasons for the softness of muscles incubated pre-rigor at 38 °C.

In the current study, it has been observed that purge loss increased with storage time and elevated pre-rigor temperature (Table 2). In accord, muscles incubated at 38 °C had the poorest WHC of myofibrils on both day 1 and 4 (Table 3). Similar results have been found previously (Liu et al., 2014; Warner et al., 2014), and the increased purge loss was suggested to be the result of reduction of unit cell volume due to the increased elastic pressure within the sarcomeres and protein denaturation caused by elevated pre-rigor temperature and fast decrease of pH (Kim et al., 2014; Offer & Knight, 1988). After myofibrils were incubated with Ca^{2+}, the WHC of myofibrils was observed to increase (Table 3) and in parallel more myofibrillar proteins were degraded (Fig. 3) and the particle size of myofibrils became smaller (Table 3), indicating that the increased WHC of myofibrils is associated with greater proteolysis caused by myofibril-bound calpains during storage. This suggests that proteolysis to some extent has the potential to counteract the detrimental effect of protein denaturation on WHC as a result of high pre-rigor temperature. Similarly, WHC was reported to be improved by proteolytic degradation of myofibrillar proteins (Huff-Lonergan & Lonergan, 2005; Kristensen & Purslow, 2001). Degradation of cytoskeletal proteins has been suggested to improve water-holding by 1) removing the linkage between myofibrils and sarcolemma followed by releasing the strain that causes outflow of water and subsequently allowing the expelled water to flow back into the muscle fiber (Kristensen et al., 2001), 2) by removing the restrictions on myofibrils in the area of the *Z*-disc and thereby allowing swelling of the filament lattice as water flows inside myofibrils (Zeng, Li, & Ertbjerg, 2017) and 3) by disrupting drip channels and entraps water in meat (Farouk, Mustafa, Wu, & Krsinic, 2012).

5. Conclusions

High pre-rigor temperature elevates the sarcoplasmic Ca^{2+} level and increases purge loss, and decreases WHC of myofibrils and shear force. With increased temperature, translocation of calpain-1 from the sarcoplasm to the myofibrils is promoted. After incubation of myofibrils with Ca^{2+}, desmin and troponin T degradation, and myofibril fragmentation caused by bound calpains increase by the order 800 μM Ca^{2+} > 40 μM Ca^{2+} > no Ca^{2+}, suggesting both calpain-1 and calpain-2 bind to myofibrils and maintain proteolytical potential. The WHC of myofibrils become greater when myofibrils are incubated with 800 μM Ca^{2+} than without Ca^{2+}, showing that proteolysis by myofibril-bound calpains causes an increase of the WHC of myofibrils. During storage, the proteolysis in muscle incubated pre-rigor at 22 and 30 °C is higher than in those incubated at 14 and 38 °C. In addition, in muscle incubated pre-rigor at 38 °C, although the amount of the enzyme measured in western blot is greater, the myofibril-bound calpain-1 activity appears to be much lower than in muscles incubated pre-rigor at lower temperatures. These results indicate that postmortem proteolysis is improved by moderately higher pre-rigor temperature (22 and 30 °C) incubation, while substantially limited by very high pre-rigor temperature (38 °C), through influence of temperature on the content and activity of myofibril-bound calpains. Additionally, it is speculated that calpain-2 is early activated and binds to myofibrils owing to a greater sarcoplasmic Ca^{2+} level caused by higher pre-rigor temperature. Calpain-2 has after binding to myofibrils proteolytical potential to degrade myofibrillar proteins at sufficient elevated Ca^{2+} level.

CRediT authorship contribution statement

Jian Lyu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Eero Puolanne:** Conceptualization, Writing – review & editing, Supervision. **Per Ertbjerg:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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.

Data availability

Data will be made available on request.

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