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Effect of LTLT heat treatment on cathepsin B and L activities and denaturation of myofibrillar proteins of pork

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ABSTRACT

The aim was to study biophysical and chemical changes during low-temperature long-time (LTLT) heat treatment of pork by measuring cathepsin B+L activity, surface hydrophobicity of myofibrils, particle size of myofibrils and effect on meat toughness as indicated by Allo-Kramer shear force. *Longissimus thoracis et lumborum* muscles were divided into large pieces, vacuum packaged and cooked in water baths at 53, 58, 63, 68 and 73 °C for 1, 8 and 24 h. The results showed that the meat toughness was markedly lower at temperatures of 53 °C and 58 °C and decreased with increasing holding time. Myofibrillar surface hydrophobicity increased with temperature, but not with time, indicating aggregation and/or gelation phenomena took place. Treatments with the lowest shear force values generally had smaller particles and were associated with high cathepsin B+L activity. A mechanism by which these cathepsins might affect the aggregation dynamics and change the mechanical properties of meat is proposed.

1. Introduction

Low-temperature long-time (LTLT) cooking has long been known to produce meat of a high eating quality. The process is mild enough to allow meat to reach thermal equilibrium with the heating medium, ensuring uniform cooking, as well as more control over the doneness of the final product. This means that, once an adequate combination of temperature and time are set, LTLT cooking often produces tender and juicy meat, regardless of the origin of the raw meat in terms of species, type of muscle or age of the animal (for a review see: Dominguez-Hernandez, Salaseviciene, & Ertbjerg, 2018). The consistency of the results has caused LTLT cooking to gain popularity and acclaim both in the food industry and home cooking (Baldwin, 2012). This thermal process is characterised by a slow heating rate, low end-point temperatures and extended isothermal heating times. The effects of this combination of variables on meat proteins have been investigated using Differential Scanning Calorimetry (DSC), and a reduction in the denaturation temperature of proteins like collagen has been shown (Christensen et al., 2013; Mitra, Rinnan, & Ruiz-Carrascal, 2017). A progressive degradation of various proteins over the duration of the thermal treatment has been observed, suggesting slow rates of myofibrillar protein denaturation at low cooking temperatures (Mitra et al., 2017; Zielbauer, Franz, Viezens, & Vilgis, 2016). Many authors have measured proteolytic activity of different cathepsins and other heat-stable proteases, and suggested proteolysis as an additional mechanism to thermal denaturation in modification of the protein structure during LTLT cooking (Christensen et al., 2013; Christensen, Ertbjerg, Aaslyng, & Christensen, 2011; Kaur, Hui, & Boland, 2020; Laakkonen, Sherbon, & Wellington, 1970). Heat treatment of pork at 55 °C results in rapid inactivation of calpain 1 and calpain 2, whereas cathepsin B and L remains active for 24 h at this temperature (Ertbjerg, Christiansen, Pedersen, & Kristensen, 2012). Cathepsin B and cathepsin L can degrade various myofibrillar proteins such as titin, nebulin, myosin heavy chain, troponin-T and troponin-I (Mikami, Whiting, Taylor, Maciewicz, & Etherington, 1987), and release of cathepsin B and cathepsin L activity from lysosomes in sous vide cooking has been observed in beef (Yin et al., 2020). Other studies have focused on connective tissue, where proteolytic activity has been linked with enhanced solubilisation of collagen and increased tenderness in LTLT cooked meat (Laakkonen et al., 1970; Roldán, Antequera, Martín, Mayoral, & Ruiz, 2013).

For the myofibrils, enzyme activity during long time thermal processing has been associated with decreased mechanical strength in beef (Christensen et al., 2013; Yin et al., 2020), and in pork (Becker, Boulaaba, Pingen, Röhner, & Klein, 2015). Cathepsin activity has been implicated in cooking-induced shrinkage of beef muscle fibres (Vaskoska et al., 2021) as well as increased myofibrillar fragmentation and

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sarcomere degradation (Yin et al., 2020). The specific mechanisms behind myofibrillar changes and the role of thermally stable cathepsins B and L are not well understood, and there are indications that they could be more complex than for collagen and essentially different to myofibrillar degradation under cold-storage (Li et al., 2019). While initial myofibrillar protein unfolding could enhance proteolysis, the progressive denaturation that occurs under extended isothermal heating could eventually inhibit enzymatic action (Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008) and further tenderisation would be impaired. Also, it remains a question why some LTLT cooking treatments do not exhibit the toughening often associated with extensive denaturation of myofibrillar proteins, such as actin, and if this effect is associated with proteolytic action. Most evidence available on myofibrillar protein degradation, unfolding and aggregation during LTLT cooking is limited to the common process temperatures of 53 and 58 °C (Li et al., 2019; Mitra et al., 2017). Only few studies have investigated the impact of extended holding time at cooking temperatures up to 74 °C on the kinetics of denaturation, and mainly in relation to cooking loss (Berhe, Engelsen, Hviid, & Lametsch, 2014; Zielbauer et al., 2016). Therefore, more information is needed about the steps involved in the denaturation process that occurs during extended isothermal heating and their influence on tenderness development. The aim of this study was to investigate the impact of a 24 h isothermal heating period under a wide range of cooking temperatures (53 to 73 °C) on biophysical changes that occur in pork myofibrils as they denature, in relation to residual cathepsin B and L activities and toughness of cooked meat.

2. Materials and methods

2.1. Samples and thermal treatments

Longissimus thoracis et lumborum muscles were obtained from both sides of 6 slaughter pigs at 48 h post-mortem after conventional slaughter and cooling procedures at a commercial slaughterhouse in Finland. The muscles (12 in total) were taken from the 5th thoracic vertebrae to the last lumbar vertebrae and showed no signs of being PSE (pale, soft and exudative) or DFD (dark, firm, dry). Muscles were trimmed of visual connective tissue and external fat, vacuum packaged, and chill stored at 4 °C for 3 additional days and subsequently cut into pieces along the muscle (8 per muscle, 6 cm in length). Each piece was weighed, numbered, individually vacuum packaged and frozen at −20 °C until processing.

A full factorial design with 15 treatments (5 temperatures and 3 cooking times) was used for the cooking experiment. The treatments were randomly assigned to samples from each animal, so each was repeated six times in total. Before cooking, the previously packaged samples were placed in a cold room at 4 °C to defrost overnight. Once thawed, they were immersed in pre-heated water baths at 53, 58, 63, 68 or 73 °C, and the cooking time (1, 8 or 24 h) was measured from the moment thermal equilibrium between the heating medium and the sample was reached (max. 1.3 h, according to preliminary tests). The cooked meat was removed from the baths once the cooking time was completed and then cooled down to 4 °C in iced water before opening the bags.

Cook loss for cathepsin B+L activity analysis was collected from the cooking bags after the meat was removed, placed in falcon tubes and centrifuged at 5100 ×*g* for 15 min at 4 °C before freezing at −80 °C. Subsamples of cooked meat for myofibrillar protein extraction and analysis were packed in aluminium foil and frozen at −80 °C. Cooked meat intended for mechanical tests was placed in new plastic bags and left overnight in a cold room (5 °C) and processed the next day.

2.2. Mechanical tests

From each cooked meat sample, 4 to 8 slices of 20 × 20 × 6 mm (longest dimension parallel to fibre axis) were cut and weighed. Then each cube was placed in an Allo-Kramer shear cell with 10 blades and cut across the fibre axis with an Instron Model 6625 (Instron Co., Canton, MA) using a load cell of 5 kN and a speed of 50 mm per min. The average value was calculated as the shear force ($N \cdot g^{-1}$) of each meat piece.

2.3. Myofibril extraction and protein quantification

The conditions for myofibril extraction were adapted from the method presented in Liu, Puolanne, and Ertbjerg (2014). Briefly, 2 g of the frozen samples were homogenised at 13,500 rpm (IKA Ultra-Turrax T25 basic; Labortechnik, Staufen, Germany) in centrifuge tubes with 10 mL ice-cold rigor buffer (75 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA, pH 7), and centrifuged 10 min at 10,000 ×*g*). The supernatant was discarded and replaced with 10 mL of cold rigor buffer. The extraction process was repeated, using a longer centrifugation cycle (20 min), to wash off the sarcoplasmic proteins. The myofibril pellet thus obtained was re-suspended in 10 mL cold rigor buffer by homogenisation. All pellets were stored at −20 °C until analysis. Protein content was determined by diluting pellet samples in 1% SDS before determination using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Samples were run in triplicates; the protein content was the calculated average of the results.

2.4. Protein denaturation

2.4.1. Surface hydrophobicity

Myofibrillar protein denaturation after LTLT treatments was monitored by measuring the amount of bromophenol blue (BPB) that was bound to myofibrils. Increases in bound chromophore are indicative of unfolding of myofibrillar proteins and exposure of hydrophobic groups at the surface of the protein structures. This measurement can thereby give information on the heat-induced denaturation of myofibrillar proteins that occurs during the LTLT treatment. The method was as described by Chelh, Gatellier, and Santé-Lhoutellier (2006). Extracted myofibrils were resuspended in rigor buffer and one millilitre of the suspensions was diluted to a protein content of 5 mg/mL, and then mixed with 200 μL of a 1 mg/mL BPB solution. The mixture was incubated for 10 min in the dark at room temperature, and finally centrifuged (5 min at 12,000 × g). An aliquot of the supernatant was diluted 10 times in rigor buffer and the absorbance at 595 nm was measured. Analyses were performed in triplicate, and the surface hydrophobicity was expressed as μg BPB bound per mg myofibrillar protein.

2.4.2. Particle size

For a complementary protein denaturation measurement, a subset (representative subsample) of the cooked meat was selected (3 samples for each time and temperature combination) and used in particle size analysis as a proxy for protein aggregation (Mitra et al., 2017; Promeyrat et al., 2010). The sizes of meat particles in an aqueous dispersion were determined by laser diffraction in a Malvern Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). The method used was similar to the one presented by Lametsch, Knudsen, Ertbjerg, Oksbjerg, and Therkildsen (2007), but no filtration or centrifugation processes were used. Samples (2 g) of cooked and raw meat (for reference, 3 samples) were homogenised in falcon tubes using 20 mL extraction buffer (100 mM KCl, 20 mM K_2HPO_4, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM NaN_3, pH 7) at 13500 rpm (2 × 20 s) and at 0 °C to minimise protein changes. This homogenisation speed and time was estimated to create particle sizes suitable for the laser diffraction analysis and still enable the heat treatment effects to be reflected in the data. Visible pieces of connective tissue were removed from the tubes and all samples were analysed immediately after homogenisation. The measurements were performed using the wet sample dispersion unit of the Mastersizer, using distilled water as dispersant (approximately 800 mL, room temperature). The model used for calculations assumed non-spherical, opaque particles

with a refractive index of 1.46 and absorption coefficient of 0.01, and dispersant refractive index of 1.333. During the measurements, constant flow of particles through the measuring cell and good background quality (≥75%) were ensured (15–20% laser beam obscuration). Distilled water was added for cleaning until the laser beam obscuration was 0% before each sample was analysed. Six measurements (10 s each) were performed for each sample homogenate and results are presented as the average values of those measurements. The parameters reported were Dx(50) to provide information on the mean diameter of the analysed particles, as well as Dx(90) and D[4,3] which are related to the presence of aggregates in suspension. Differential size distributions curves of cooked pork were obtained to show the dynamics of particle size over the studied cooking temperatures and times.

2.5. Cathepsin B+L activity

The combined residual activity of cathepsin B and L in the cook loss was measured by observing the increase in product concentration over a set time (end-point assay). The fluorimetric assay procedure was as described in Christensen et al. (2011). Prior to the assay, the collected cook loss was thawed, and each sample (15 μL) was mixed with 135 μL of activation buffer (340 mM sodium acetate, 60 mM acetic acid (100%), 4 mM EDTA, 0.1% Brij 35 (30%) and 8 mM dithiothreitol, pH 5.5) and incubated at 40 °C with 100 μL substrate (12.5 μM *Z*-Phe-Arg-NMec, Sigma-Aldrich, Saint Louis, USA) for 10 min. The reaction was arrested by adding 1 mL stop buffer (100 mM NaOH, 30 mM sodium acetate, 70 mM acetic acid, 100 mM trichloroacetic acid, pH 4.3). The incubated samples were always kept from light. Incubated sample (250 μL, in duplicates) were added to each well in black, flat bottomed microtiter plates, and fluorescence was measured at 355 nm/460 nm (excitation/emission) in a Tecan Infinite M200 plate reader (Tecan, Austria). The amount of 7-amino-4-methyl coumarin (AMC, Sigma) contained in the wells was obtained directly from a standard curve prepared using dilutions of AMC and stop buffer. One milli-unit (mU) of cathepsin B+L activity equals to the release of 1 nmol of AMC per minute of reaction at 40 °C; activity results were expressed in μU/mL cook loss.

2.6. Statistical treatment

Statistical treatment of raw data was performed using the General Linear Model in Minitab 17 (Minitab Inc., State College, PA, USA). The effect of the different heat treatments was evaluated on Allo-Kramer shear force, residual activity of cathepsin B+L, surface hydrophobicity and particle size parameters of LTLT cooked meat. Diameter values and residual activity were log-transformed to improve normality (Huston & Deming, 2002). Time and temperature were treated as fixed effects, using the replicate (animal) as the blocking factor. In the ANOVA, effects were considered significant with $P \leq 0.05$. Least square means were compared using a Tukey test with 95% confidence. Relation between the measured variables was evaluated on untransformed values using Spearman-Rho correlation coefficients ($\alpha = 0.05$).

3. Results

The combined effects of time and temperature during LTLT cooking of *longissimus thoracis et lumborum* were investigated. Table 1 shows that heating time and temperature significantly affected shear force and cathepsin B+L activity, while surface hydrophobicity and particle size parameters were significantly affected only by temperature. Time and temperature showed significant interaction on shear force and cathepsin B+L activity. During the first hour of heating, the Allo-Kramer shear force declined with increasing temperature, followed by an increase in the range between 63 and 73 °C (Fig. 1). Increasing the cooking time from 1 to 8 h decreased the shear force especially at 58 °C. No clear effect on toughness of increasing the heating time from 8 h to 24 h was observed.

Table 1
Significant main effects of temperature, time and their interaction for the studied traits. Particle size is represented by Dx(50), Dx(90) and D[4,3] values.

Trait	Temperature	Time	Temperature × Time
Allo-Kramer shear force	<0.001	<0.001	0.001
Cathepsin B+L Activity	<0.001	<0.001	<0.005
Surface Hydrophobicity	<0.001	0.36	0.56
Dx(50)	0.005	0.92	0.35
Dx(90)	<0.05	0.26	0.74
D[4;3]	<0.05	0.32	0.74

P values for cathepsin activity, Dx(50), Dx(90) and D[4,3] correspond to log-transformed data.

The residual activity of cathepsin B+L was largest at the combination of low cooking temperatures of 53 °C to 58 °C and one hour of cooking, indicative of heat activation of the enzymes. Further heating (either an increase of temperature or time) resulted in a concurrent decline of the activity, although about 20% of the maximum activity remained even at the most severe treatment (73 °C for 24 h). The increase in surface hydrophobicity was generally related to the increase in temperature at levels above 63 °C (Fig. 1 C), with the values remaining essentially constant during the entire range of heating time from 1 to 24 h. A similar trend was observed in the particle size analysis. The particle size reflects aggregate formation in the myofibrillar structure occurring during cooking but may to some extent also show enzymatic weakening due to proteolytic processes. As can be seen in Table 2, the diameters Dx(50), Dx(90) and D[4,3], also known as D-values, increased with heating intensity (in relation to temperature), so meat cooked at 73 °C presented larger sizes than meat cooked at lower temperatures and raw meat.

The particle size distribution curves of raw and cooked whole meat suspensions are shown in Fig. 2, for size classes between 1.3 μm and 3 mm. Raw meat presented a clearly tri-modal distribution whereas LTLT cooked meat was essentially bi-modal. The temperature-related size increase observed in the diameters could be also appreciated in Fig. 2, as the reduction in number of particles located on the first (1.3–29 μm) and second (33–260 μm) modes of the distributions, as well as the appearance of shoulders on the larger size peak (290–1200 μm) right side. The behaviour over time was variable among the studied temperatures. Increasing time, from 1 to 24 h, between 58 and 68 °C produced a decrease in shoulder prominence, while cooking at the extremes (53 and 73 °C) for the same duration generally increased the amount of coarse particles.

As can be seen in Table 3, there were significant moderate correlations between some of the parameters. Cathepsin B+L activity showed negative correlations to shear force, surface hydrophobicity and the particle size, while shear force was positively correlated to particle size parameters. Surface hydrophobicity also showed positive correlations with particle size.

4. Discussion

A variety of physicochemical changes, in relation to different time and temperature combinations, were studied in the present work, aiming to better understand the mechanisms behind the desirable tenderness obtained during LTLT cooking of meat. Denaturation of myofibrillar proteins, measured as surface hydrophobicity and particle size was mainly governed by the end-point temperature during LTLT. Previous studies measuring denaturation with the BPB probe on isolated myofibrils showed that the amount of exposed hydrophobic sites over short time cooking increased from 50 °C to 60 °C (Chelh et al., 2006). This is consistent with our results and is in accordance with the exposure of aromatic residues and the increasing polar environment produced by cooking (Berhe et al., 2014). The lack of measurable change in surface hydrophobicity at longer cooking times and higher temperatures (above 60 °C) could be indicative of hydrophobic residues being involved on aggregation or gelation (Santé-Lhoutellier et al., 2008; Tornberg, 2005).

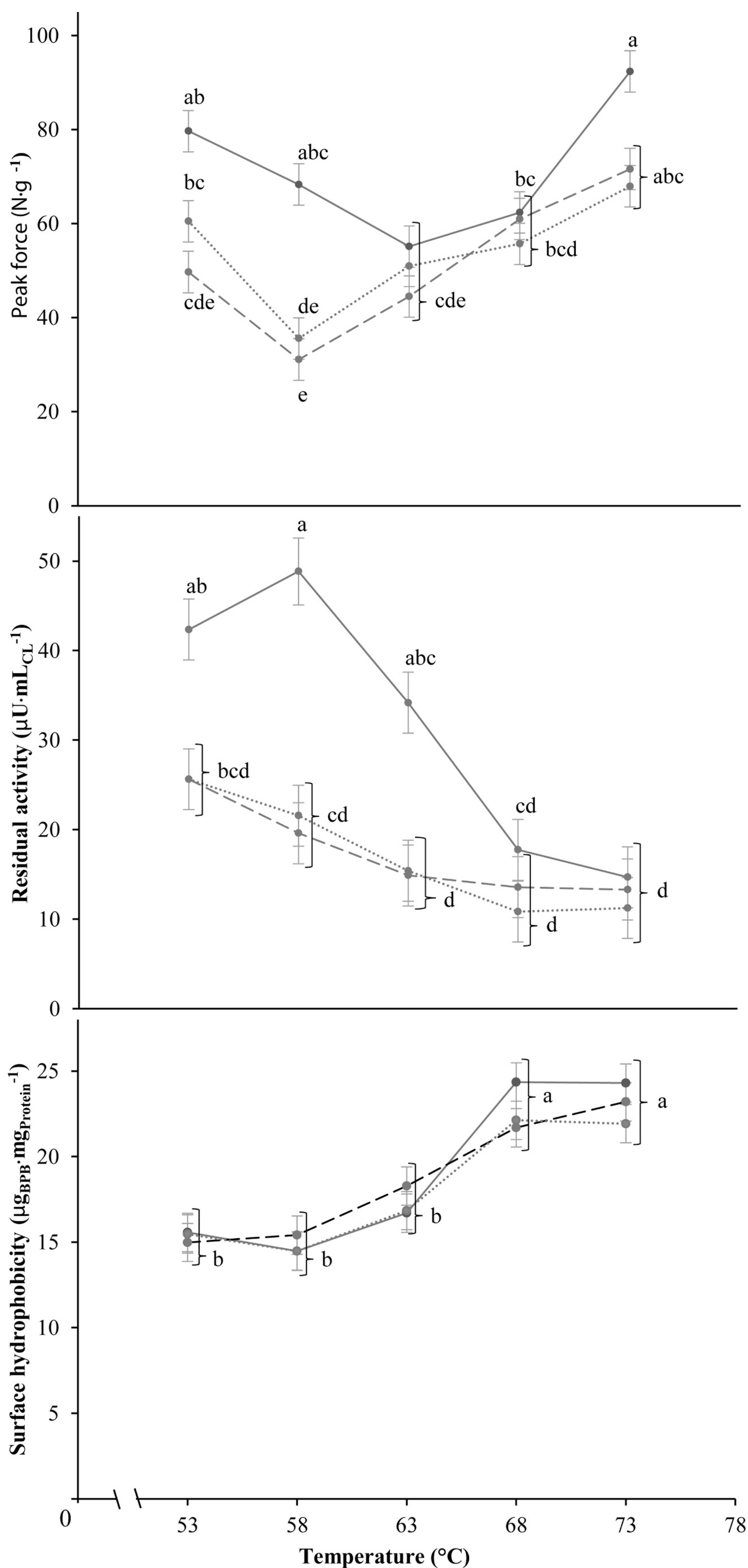

Fig. 1. Fitted means (n = 6) of Allo-Kramer shear force, residual cathepsin B+L activity and surface hydrophobicity for porcine *longissimus thoracis et lumborum* cooked at different temperatures and holding times: 1 h (—), 8 h (···) and 24 h (—). Bars represent standard error of the means. Letters refer to significance according to a Tukey test (α = 0.05). Heat treatments that do not share a common letter differ. For residual activity, comparisons were done on log-transformed values, but means are reported as untransformed values.

Table 2
Fitted means of particle size diameters obtained for porcine *longissimus thoracis et lumborum* ($n = 3$) raw and cooked at different temperatures.

Diameter	Unit	Raw	Temperature (°C)					SEM
			53	58	63	68	73	
Dx(50)	μm	56	83[a]	91[ab]	93[ab]	96[ab]	107 [b]	3.8
Dx(90)		154	285[a]	344[ab]	356[ab]	367[ab]	418[b]	29
D[4;3]		95	124[a]	140[ab]	143[ab]	146[ab]	166[b]	9.1

Dx: fraction of the sample (50% or 90%), that is finer to the denoted size. D[4,3]: mass to volume mean diameter. SEM: standard error of the mean. Letters refer to significance between cooked samples according to a Tukey test ($\alpha = 0.05$) on log-transformed data; means with the same letter do not differ. Values of raw samples are included as reference.

This is supported by the correlation between increasing particle sizes and surface hydrophobicity (Table 3).

Other authors have studied aggregation of meat proteins upon heat treatment using particle size measurements (Mitra et al., 2017; Promeyrat et al., 2010; Sun, Zhou, Zhao, Yang, & Cui, 2011), detecting an increase of size with temperature when compared to the raw product. Similar trends were obtained here (Table 2).

Even though comparing particle sizes based only on summary curve parameters (such as D-values) can be useful, it might not be enough to detect differences between the shapes of particle size distribution curves (Scheibelhofer, Besenhard, Piller, & Khinast, 2016). This could explain why the present study (Table 2) and previously reported D-values of cooked meat (Mitra et al., 2017) were only able to detect significant differences between the mildest and the most severe treatments. However, the trends of the particle size distribution curves over the range of assayed temperatures and times showed great variability (Fig. 2). According to Soglia et al. (2017), the peaks in a raw meat particle size distribution curve correspond to different components, so the larger fraction could contain a mixture of long myofibril fragments and extracellular matrix, while the small and intermediate sized fraction could be mainly composed by myofibrils and their fragmentation products (Lametsch et al., 2007). Upon cooking, the loss of a distinguishable third peak is likely related to denaturation and reduction of integrity in the myofibrils and solubilisation of collagen observed in LTLT at or above 50 °C (Christensen et al., 2011), whereas the overall changes in shape (Fig. 2 B–F) could be related with changes in interprotein interactions.

At higher temperatures and longer cooking times, the particle size distribution dynamics showed a behaviour similar to the progression of aggregation as described by Promeyrat et al. (2010) and Morris, Watzky, and Finke (2009). At 68 °C, the shoulder reductions (Fig. 2 E) relates to the folding of elongated aggregates to form compact circular or amorphous ones, and then, at 73 °C, the increase in size could be attributed to polymerisation forming bigger and heavier particles (as attested by the D[4,3] values and the particle size distribution in Fig. 2 F). However, this explanation cannot be applied for treatments at lower temperatures, where results showed that the particle size remained essentially constant or increased with temperature (Table 2), but not with time (Fig. 2 B, C and D). The particle size distribution behaviour seemed to indicate that as cooking time increased between 53 and 63 °C, no coarse aggregates were formed, and smaller particles were detected (Fig. 2 A), suggesting that other factors besides denaturation were involved.

Residual activity of cathepsin B+L has been found to be heat stable under a wide range of LTLT conditions, up to 70 °C and 1 h, in both pork (Ertbjerg et al., 2012) and beef (Kaur et al., 2020). These enzymes are more thermally stable than the calpains, whose activity is lost quickly in the range of LTLT cooking temperatures (Christensen et al., 2011; Ertbjerg et al., 2012). In the present work cathepsin B+L residual activity was retained even at the most severe treatment (73 °C and 24 h), which could be associated to the length of ageing of the meat used. Proper ageing not only weakens the fibres prior to heating (Li et al., 2019), it also allows cathepsins B and L (and other thermally stable lysosomal proteases) to be released into the sarcoplasm, diffuse into the fibres, and be active or even activated during the first part of cooking (Christensen et al., 2011; Ertbjerg et al., 2012). In this context, proteolytic activity affecting thermal denaturation of myofibrils during LTLT cooking has been theorised (Christensen et al., 2013; Zielbauer et al., 2016), but the underlying mechanisms have not been further investigated, and it has been merely suggested that cathepsins B and L could cause structural weakening and lower the temperatures required for denaturation, as seen in previously reported DSC thermograms (Christensen et al., 2013).

The results of the present study show for the first time the existence of differences in aggregation dynamics between heat treatments with high and low enzymatic activity, and that negative correlations existed between residual cathepsin B+L activity, surface hydrophobicity and the presence of large aggregates of myofibrillar proteins (Table 3). We hypothesise that during the first hours of cooking at ≤63 °C, the release and activation of cathepsins B and L would increase the probability of proteolytic attack and counteract the formation of large aggregates as cooking time increase, thus explaining the trends in particle size distribution (Fig. 2). At these mild heat treatments (short time and low temperature) rates of denaturation would be slow and aggregates might be easily disrupted if the main interaction bonds are electrostatic or hydrophobic (van der Linden & Venema, 2007). Unfolding is likely to enhance hydrolysis by cathepsins B and L, by opening the structure of proteins and exposing buried hydrophobic chains and substrate cores (Polajnar et al., 2013). Yin et al. (2020) reported that cathepsin B+L activity activity at mild LTLT treatments was associated with myosin heavy chain hydrolisation and smaller myofibrillar fragments, and this would likely result in less protein aggregation at extended cooking times. A similar role of proteases influencing aggregation has been suggested during thermal processing of muscle products (Sun et al., 2011), and this explanation is in accordance with the functionality of cathepsins B and L in living tissues, where they act as a defense system against the formation of polymerised aggregates (Perlenfein & Murphy, 2017; Polajnar et al., 2013).

When treatments at 68 and 73 °C are examined, interactions would occur at higher rates, favoured by further changes in secondary structures and intermolecular disulphide bonds (Beattie, Bell, Borggaard, & Moss, 2011; Berhe et al., 2014). These changes have been regarded as essential in the formation of pork protein aggregates and stronger gels (Liu, Zhao, Xie, & Xiong, 2011). Thus, more stable aggregates would form and, once a sufficient concentration is reached, start polymerising into large ones (Morris et al., 2009). In these conditions, any proteolysis by the remaining active proteases would likely be impaired by the poor substrate quality of the formed aggregates (Grune, Jung, Merker, & Davies, 2004; Santé-Lhoutellier et al., 2008).

The changes in aggregation would reflect on the meat texture. Thermally induced protein-protein association determines the number of junction points stabilising and strengthening the structures of cooked meat as it transforms from a viscous to a more elastic gel (Xiong & Brekke, 1990). In the present investigation, higher toughness values were associated with the presence of coarse aggregates (Table 3), but two distinguishable temperature trends for the changes in mechanical properties, possibly reflecting high (between 53 and 58 °C) and low (68 and 73 °C) enzyme activity were also observed.

The main implication of these findings is that they point to a possible mechanism by which myofibrillar proteolysis during cooking affects the outcomes of the LTLT process. We hypothesise that Cathepsin B+L activity during the first hours of cooking below 63 °C interfered with the natural progression of thermal unfolding-aggregation phenomena in myofibrillar proteins, affecting subsequent gel formation and thus produced fragile structures at the later stages of heating. This tenderising effect could be enhanced by sarcoplasmic protein precipitating between myofilaments making crack propagation in the gel easier (Tornberg, 2005), but also by a heat-induced reduction of the strength of connective

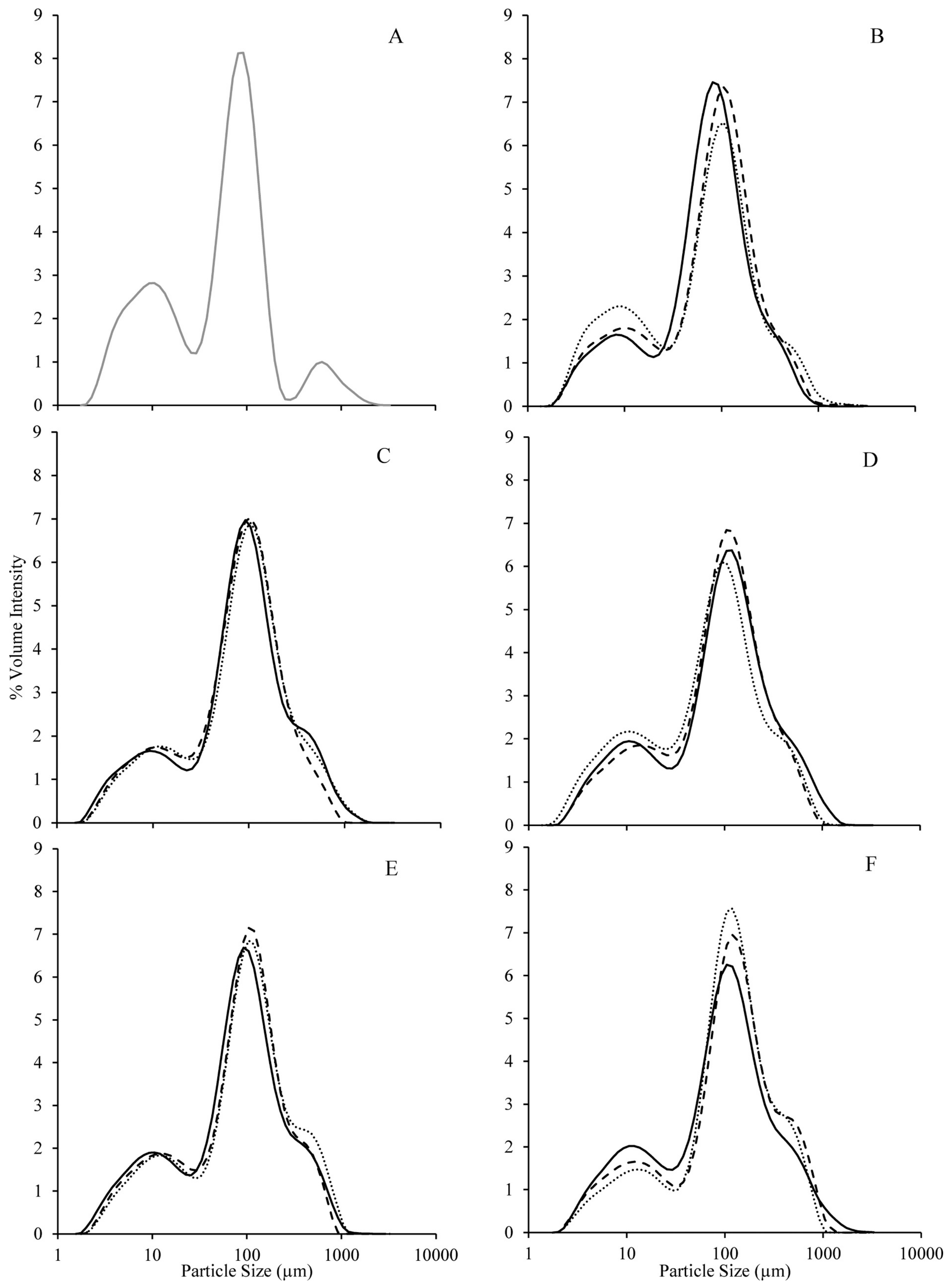

Fig. 2. Particle size distributions of raw (A) and LTLT cooked (B: 53 °C, C: 58 °C, D: 63 °C, E: 68 °C and F: 73 °C) porcine *longissimus thoracis et lumborum*. Holding times for cooked meat: 1 h (—), 8 h (···) and 24 h (—).

Table 3
Correlations between selected biophysical parameters of LTLT cooked porcine *longissimus thoracis et lumborum*. Particle size is represented by Dx(50), Dx(90) and D[4,3] values.

Parameter	Allo-Kramer shear force	Cathepsin B+L activity	Surface hydrophobicity
Cathepsin B+L activity	−0.53 *		
Surface hydrophobicity	0.50 NS	−0.74 **	
Dx(50)	0.43 NS	−0.77 ***	0.56 *
Dx(90)	0.50 *	−0.82 ***	0.54 *
D[4;3]	0.52 *	−0.80 ***	0.51 *

NS: not significant.
* $P \leq 0.05$.
** $P \leq 0.01$
*** $P \leq 0.001$.

tissue (Latorre, Palacio, Velázquez, & Purslow, 2019). Proteolysis could also prevent or counteract some of the toughening occurring upon extensive myofibrillar protein denaturation after prolonged heating at ≤63 °C, and also enhance protein solubility and digestibility of LTLT cooked meat (Bhat, Morton, Zhang, Mason, & Bekhit, 2020).

At higher temperatures, where proteolysis is absent or much reduced, denaturation proceeds and its effects become central in describing the properties of cooked meat, both at short and long cooking times. Above 65 °C and in less than 1 h of cooking, previous studies have shown that the myofibrillar protein fraction denatures (Zielbauer et al., 2016) and, in agreement with our results, becomes heavily aggregated (Promeyrat et al., 2010). The formation of inter-protein bonds prior to gelling would form a more elastic and denser matrix, strengthen cooked meat structure and explain the increased toughness observed in the initial phase of cooking at 68 and 73 °C. Finally, the eventual reductions in hardness at 73 °C could be attributed to the recognized effect of collagen gelatinisation but, given the increases in the proportions of mid-sized particles observed in the particle size distribution, chemical hydrolysis of the myofibrils could also play a part.

5. Conclusions

Changes in relation to tenderness and particle size were observed during LTLT cooking between 53 °C and 73 °C. Fast changes, observable at short times and/or higher temperatures, correspond to the recognized behaviour of myofibrils upon cooking, resulting in toughening as the temperature increases. At longer times and lower temperatures, a complex interplay between aggregation and proteolysis is established and could be responsible for the observed dynamics. The overall findings from this research suggest that LTLT cooking between 53 °C and 63 °C offers an opportunity to counteract the heat-induced toughening of myofibrils via the action of proteolytic enzymes, which in conjunction with the changes occurring in other protein fractions, contribute to decreasing toughness.

Authorship statement

E. Dominguez-Hernandez: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition

P. Ertbjerg: Conceptualization, Validation, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition

Declaration of Competing Interest

None.

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