Changes in Cathepsin Activity during Low-temperature Storage and Sous Vide Processing of Beef Brisket

Lovedeep Kaur[1, 2*], Seah Xin Hui[1] and Mike Boland[2]

[1] School of Food and Advanced Technology, Massey University, 4442 Palmerston North, New Zealand

[2] Riddet Institute, Massey University, 4442 Palmerston North, New Zealand

Running title: Cathepsin activity in beef brisket

Author contributions:

- Conceptualization: Kaur L.
- Data curation: Hui SX., Boland, M, Kaur L.
- Formal analysis: Kaur L.
- Methodology: Hui SX, Boland, M, Kaur L.
- Software: Hui SX.
- Validation: Hui SX, Kaur L.
- Investigation: Hui SX, Boland, M, Kaur L.
- Writing - original draft: Hui SX, Kaur L.
- Writing - review & editing: Hui SX, Boland, M, Kaur L.

---

*Corresponding author

E-mail: L.Kaur@massey.ac.nz; Tel. +64 6 951 7261

Abstract

It is believed that two main proteolytic systems are involved in the tenderization of meat: the cathepsins and the calpains. Many researchers consider the calpain system to be the major contributor to meat tenderness during post-mortem storage. However, the role and activity of cathepsins during post-mortem storage or low temperature meat processing is unclear, particularly for the tough meat cuts like brisket. Thus, the study was designed to investigate the effects of cold (refrigerated and frozen) storage and sous vide processing on the activities of cathepsin B, H and L in beef brisket. There were no significant changes in pH and cathepsin H activity throughout the 18 days of storage at both temperatures. However, an increase in cathepsin B activity was observed during the first 4 days at both storage temperatures, but subsequently the activity remained unchanged. Cathepsins B and L were found to be more heat stable at sous vide temperatures (50˚C for 24 h, 55˚C for 5 h and at 60˚C and 70˚C for 1 hour) compared to cathepsin H. Cathepsin B + L activity was found to increase after sous vide cooking at 50˚C for 1 h but decreased to about 47 % relative to the uncooked control after 24 h of cooking. These results suggest that cathepsins B and L may contribute to the improved meat tenderness usually seen in sous vide cooked brisket meat.

Keywords: Cathepsin; endogenous enzymes; meat; post-mortem storage; sous vide

1. Introduction

Meat tenderness is generally considered by the consumer to be the most important palatability factor. Post-mortem tenderisation in skeletal muscle is a complex phenomenon that has yet to be fully understood. Muscle is tender right after the animal is slaughtered but once the onset of rigor mortis, a permanent cross-link between actin and myosin proteins is formed and this leads to muscle toughness (Bowker *et al.,* 2010). Beef requires ageing for at least 14 days in a controlled environment with temperature ranging from -1 to 5˚C to achieve tenderness (Christian & Stephen, 2010). During this period, the degradation of muscle contributes to meat tenderness. This process is known as *post-mortem* aging (also known as maturation or conditioning) of meat and is widely practiced by beef producers (Cheret *et al.,* 2007; Christian and Stephen, 2010).

Sarcoplasmic proteases are crucial in protein catabolism and post-mortem muscle softening. It is believed that two main proteolytic systems are involved in the tenderization of meat: the cathepsins and the calpains. Many researchers consider the calpain system to be the major contributor to meat tenderness during post-mortem aging (Koohmarie and Geesink, 2006). However, this assumption has been debated (Herrera-Mendez *et al.,* 2006) and the role of cathepsins is not fully understood yet. Cathepsins exhibit greater heat stability than calpains Laakkonen *et al.,* 1970; Pomponio and Ertbjerg, 2012) as the latter have been reported to completely inactivate at temperatures above 55 ℃ whereas cathepsins, particularly B and L have been reported to remain active even after 24 hours of heating at 55 ℃ (Christensen *et al.,* 2011; Ertbjerg *et al.,* 2012). Cathepsin D has also been reported to have a lower heat resistance compared with cathepsin B and L (Spanier *et al.,* 1990). Cathepsin B and L are endopeptidases that may contribute to meat tenderness by weakening collagen in connective tissue, leading to its increased solubility (Agarwal, 1990; Christensen *et al*., 2013; Solvig, 2014). Incubation of connective tissue with cathepsin B has been reported to significantly decrease the denaturation

78 temperature of connective tissue from both calf and steer (Beltrán *et al*., 1992). In addition, a
79 study by Burleigh *et al*. (1974) has shown that cathepsin B contributes to the degradation of
80 both soluble and insoluble collagen by eliminating intermolecular cross-links.
81
82 In addition to proteolysis during ageing, meat tenderness may continue to develop during the
83 cooking step, particularly when the meat is heated for a longer duration at temperature that is
84 optimum for enzyme activity (Ertbjerg *et al.*, 2012). Sous vide is a method of cooking vacuum-
85 packaged food at a precise temperature for a long duration, from hours to days (Baldwin 2012).
86 This form of cooking helps to retain moisture and is known to produce tender and juicy meat
87 (Laakkonen *et al.*, 1970). Temperatures from 55 to 80˚C and cooking for 6 to 48 hours are
88 typical conditions for cooking meat (Baldwin, 2012).
89
90 We hypothesized that cathepsins would remain active during sous vide cooking and could still
91 contribute to the tenderization process of beef brisket during the cooking process as opposed
92 to cooking meat at high heat such as grilling whereby high cooking temperature and time is
93 often associated with the toughening of meat. Limited studies have been done to examine the
94 effects of sous vide cooking on the proteolytic enzyme activities in tough beef muscle cuts such
95 as brisket. Thus this investigation was designed to study the effects of (1) *post-mortem* storage
96 and (2) sous vide cooking at different temperatures (50-70˚C) for up to 24 h on the activities
97 of the B, L and H cathepsins in beef brisket, which will aid in determining the contribution of
98 these cathepsins to tenderness of tough cuts of meat.
99
100 **2. Materials and Methods**
101 *2.1. Materials*
102 All chemicals used in the study were of analytical grade.

2.2. *Muscle samples and preparation*

Hot-boned briskets from three steers at 4 hours *post-mortem* were kindly provided by a local slaughterhouse (ANZCO Foods, Bulls, New Zealand) and immediately transported to the laboratory. At approximately 6 hours *post-mortem*, muscles were cut into small samples of 2 cm^3 or thin strips after removal of the visible subcutaneous fat, vacuum packed into portions and stored in either 4˚C or -20 ˚C. Post-mortem storage and sous vide experiments were two separate experiments that were run in parallel, with two different objectives, but with the samples from the same carcasses. Thus, for each carcass, the samples were divided into three batches as described in Figure 1: as control, and for *post-mortem* storage and sous vide experiments. Three samples per treatment from different carcasses were analysed for pH and cathepsin B and H activities as described in sections 2.5 and 2.6.

2.3. *Post-mortem storage experiments*

The experiment to assess the effects of *post-mortem* storage conditions on cathepsin activities was divided into short-term (6 hours to 4 days *post-mortem* at either 4˚C or -20 ˚C) and long-term storage (further 4-18 day storage at either 4˚C or -20 ˚C of the 4 day refrigerated meat). The vacuum-packed muscle samples were stored in a chiller or freezer at 4˚C and -20 ˚C, respectively. At the end of the allocated storage time (Figure 1), samples were assayed for pH and cathepsin activities.

2.4. *Sous vide experiments*

One-day post-mortem meat stored at 4℃ was chosen for performing the sous vide experiments, based on previous reports (Chéret *et al*., 2007). Slight modifications were made to the sample preparation procedures reported by Ertbjerg, Christiansen, Pedersen, and Kristensen (2012) Meat was cut into small strips, vacuum packed and stored at 4˚C on the day of slaughter (Figure

1). At one day *post-mortem*, the vacuum bags containing the meat strips were cooked in water baths set at 50, 55, 60, 65 and 70 ˚C for 1, 5 or 24 hours. At the respective time interval, samples were removed from the water bath and cooled in ice water to below 25 ˚C. The temperature of the water bath was monitored using a digital thermometer (Q1437, Dick Smith Electronics) to ensure that the desired temperature had been reached before fully submerging the bags into the water bath. Since the samples were cut into thin strips and as confirmed from previous experimentation (Zhu *et al*., 2018), it was assumed that the core temperature of the samples reached the water bath temperature quite quickly (in less than an hour).

Three random cooked samples from each carcass were homogenised together and assayed for pH and cathepsin activities. All samples were stored at -20 ˚C until analyzed. Cathepsin activities were calculated for both cooked and raw samples and expressed as a relative activity (%):

$$(A_t/A_0) \times 100$$

where A_t and A_0 are the enzyme activities of cooked and raw samples, respectively.

2.5. pH determination

The pH of the meat homogenates were determined with a glass electrode pH meter (Cyberscan pH 510, Eutech Instruments). The pH meter was calibrated using pH 7.0 and 4.0 standard buffers stored at room temperature. Meat homogenate was prepared by blending finely-chopped meat with milli-Q water in a ratio of 1:10 for 1 min using a food processor (BFP100WHT, Breville).

2.6. Preparation of sarcoplasmic protein extract

Sarcoplasmic protein extract was prepared using the method described by Chéret *et al.* (2007) with slight modifications. Three random muscle samples from a single carcass were finely

chopped and homogenized with an extraction buffer comprising of Tris–HCl, 2-mercaptoethanol and ethylenediaminetetraacetic acid in a ratio of 1:3 for 1 min using a food processor (BFP100WHT, Breville). The homogenized mixture was centrifuged at 25,000 × *g* for 20 min at 4 ˚C in a Sorvall Evolution RC centrifuge (Thermo Fisher Scientific, USA). The supernatant was collected and filtered using 0.45 µm syringe filter and referred to as crude extract. This crude extract was immediately used for the cathepsin assays.

2.6.1. Determination of cathepsins B, H and L activities

Activities of cathepsin B, H and L were analyzed in the sarcoplasmic extract using the method described by Chéret *et al*. (2007). The cathepsin activities were determined at room temperature in a 96-well microplate, consisting of 6 µl of 5% CHAPS prepared in milli-Q water; 1 µl of 1.40 M 2-mercaptoethanol; 16 µl of 5 % (w/v) Brij® 35 prepared in milli-Q water; 5 µl of 20 mM synthetic fluorogenic substrate prepared in methanol and 70 µl of 0.4 mM acetate/acid acetic (pH 4) buffer containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The substrates for cathepsin B, cathepsin B and L and cathepsin H were Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (C5429, Sigma-Aldrich), Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (C9521, Sigma-Aldrich), L-Arginine-7-amido-4-methylcoumarin hydrochloride (A2027, Sigma-Aldrich), respectively. The reaction was initiated by the addition of 200 µl of crude sarcoplasmic protein extract. The fluorescence intensity was determined using a microplate reader (Wallace Victor2 1420 multilabel counter, Perkin Elmer) with excitation and emission wavelengths of 355 nm and 460 nm, respectively. A control was run in parallel in which the protein extract was substituted by extraction buffer. Cathepsin specific activities were expressed in FU (units of fluorescence) increase per min per g of muscle. Cathepsin L activity was calculated by subtracting cathepsin B from the cathepsin B + L activity. Cathepsin D activity could not be measured in the samples due to technical

issues in standardization of cathepsin D assay. The difficulties in standardising cathepsin D assay may be attributed by its low activity level in meat and meat products as reported by Rico, Toldrá, & Flores (1991b). Hence, this paper does not report cathepsin D activities in the samples.

2.7. Statistical analysis

Significant differences in pH and enzyme activities among different treatments were determined by one-way ANOVA Tukey test at the 95% significance level using Minitab® 17 (Minitab Statistical Software 2014).

3. Results and discussion

3.1. Effects of post-mortem storage conditions

3.1.1. Short-term storage

There was no significant difference in pH value and cathepsin H activities during 4 days of ageing at both chilled and frozen storage conditions (Table 1). Although there was no significant difference in cathepsin H activities, the numerical difference between the control and frozen samples could be because L-Arginine-7-amido-4-methylcoumarin (substrate for cathepsin H) was also cleaved by aminopeptidase, and under the conditions of the assay some residual peptidase activity may be present (Toldrá & Etherington, 1988).

For cathepsin B, there was a significant increase (compared to 6 hours *post-mortem*) in its activity after 4 days of ageing at both temperatures however no significant difference was observed among the storage temperatures (Table 1). Similar increase for cathepsin B was observed for sea bream muscles, which was attributed to enzyme activation by low pH (Matos, 2013). Cathepsin B has an optimum pH of 5.5 towards most substrates, which is near the ultimate pH of the meat. During *post-mortem* storage when the temperature and pH decrease,

the fragile membranes of lysosomes may rupture resulting in the release of cathepsins (Bowker *et al*., 2010; Lana and Zolla, 2016). Despite the fact that no significant difference was observed in the pH during 4 days of ageing, it is possible that the decrease in temperature during storage or the formation of ice crystals may have ruptured the lysosomes, releasing cathepsins, therefore contributing to an increase in cathepsin B activity.

3.1.2. Long-term storage

There was no significant change in pH value throughout subsequent 2 weeks' storage at both temperatures (Table 2). The activities of cathepsins B, L and H also remained stable and unchanged during this storage period. Similar results have been reported for ostrich fillet where cathepsin B and L showed no decrease in their activities after 12 days storage at 2 to 4 ˚C (van Jaardveld *et al.,* 1997). Previous studies have indicated that all cathepsins are capable of degrading myosin (Allen and Goll 2003) but no myosin degradation has been reported during *post-mortem* storage at 0 to 4 ˚C (Bandman and Zdanis, 1988). Moreover, the changes in shear force values during *post-mortem* ageing for 2 weeks at 1.2 ˚C for three types of bovine muscles were found to be different despite having similar level of cathepsins B, H and L (Koohmaraie *et al.,* 1988). Thus, the tenderizing effect of cathepsins during the long term storage at refrigerated temperatures remains questionable. Other proteolytic systems such as caspase, metalloproteases, thrombin and plasmin may also be involved during *post-mortem* ageing (Ouali *et al.,* 2013).

3.2 Effects of sous vide cooking conditions

3.2.1 pH

At all temperatures, increasing the cooking time did not have a significant effect on the pH (Table 3). There was a significant increase ($P < 0.05$) in pH when the temperature increased

228 from 50 ˚C to 70 ˚C, after 1 and 5 hours of cooking. An increase in pH during cooking has also
229 been observed for bovine muscles (*longissimus, semitendinosus* and *rectus femoris*) heated at
230 60 ˚C for 10 hours (Laakkonen *et al.,* 1970). Small increments in pH upon cooking of meat at
231 60 ˚C have been reported to be due to a decrease in acidic groups in the meat proteins (Hamm
232 and Deatherage, 1960).

233 3.2.2 Cathepsin activities

234 Cathepsin B + L activity was heat stable and these proteases remained active throughout
235 cooking at 50 ˚C even after 24 hours and for the first 5 hours at 55 ˚C (Figure 2). At 50 ˚C, the
236 cathepsin B + L activity increased significantly ($P < 0.05$) after 1 hour, while further cooking
237 led to a decrease in activity. There was a significant reduction ($P < 0.05$) in cathepsin B + L
238 activity after cooking at 60, 65 and 70˚C, where most of the extractable activity was lost after
239 1 hour. Subsequently no significant change ($P > 0.05$) in cathepsin B + L activity was observed
240 for meat cooked from 5 to 24 hours at these temperatures. The reduction in activity is likely to
241 be due to the heat sensitivity of cathepsin B and L, combined with the effect of the increase in
242 pH observed for these treatment conditions. A similar trend was observed for cathepsin B
243 (Figure 3) and cathepsin L (Figure 4) activity. In a study on porcine *longissimus* muscle,
244 cathepsin B + L activity was reported to increase with an increase in temperature from 48 to
245 58 ˚C (Christensen *et al.*, 2011). In another study conducted on beef *semitendinosus* muscle,
246 the activity of cathepsins B and L in the expelled cooking loss was highest after cooking at
247 53 ℃ for 2.5 hours and then decreased with increasing temperature and time (Christensen *et*
248 *al*., 2013). All these observations are consistent with our experimental results. In this present
249 study, no significant cathepsin B+L activity was detected after 5 hours heating at 60 ℃.
250 However, a higher heat tolerance of cathepsins B and L has been reported for beef
251 *semitendinosus* muscle where their activity was measurable even after 19.5 hours at 63 ℃
252 (Christensen *et al*., 2013). This could be because of the differences among the meat cuts used

253 in both the studies. Ertbjerg *et al*. (2012) found that cathepsin B + L activity reached a
254 maximum after heating at 55 ˚C for 1.5 hours in porcine *longissimus* muscle. They suggested
255 that part of cathepsin B and/or cathepsin L may exist in the form of a pro-enzyme which is
256 activated by heat. An increase in activity was also observed in our experimental results, which
257 was evident after 1 hour of heating at 50 ˚C. Increases in collagen solubilization and tenderness
258 were also evidenced in this temperature range (Christensen *et al.,* 2011). The synergistic effect
259 of heat denaturation and proteolytic action of cathepsins (B and L) has been reported to account
260 for an increased weakening effect on collagen that led to more tender meat during sous vide
261 cooking at temperatures < 55 ˚C (Dominguez-Hernandez *et al.,* 2018).
262
263 At all temperatures, a significant reduction ($P < 0.05$) of cathepsin H activity occurred during
264 the first hour of cooking (data not shown). At 50 ˚C, cathepsin H remained active (15% of
265 initial activity) during the first hour but it lost most of the extractable activity within 5 hours.
266 At 55 ˚C and above, no extractable activity was detected after 1 hour of cooking. In addition,
267 there was no significant difference ($P > 0.05$) in cathepsin H activity after 1 hour of cooking at
268 temperature ranging from 55 ˚C to 70 ˚C. Thus, it is unlikely that cathepsin H is responsible
269 for the tenderization effect usually observed during sous vide cooking of meat at temperatures
270 < 70 ˚C.
271
272 **4. Conclusions**
273 During *post-mortem* storage, only cathepsin B activity was observed to increase from 6 hours
274 to 4 days *post-mortem* at both (refrigerated and frozen) storage conditions. There were no
275 significant changes in cathepsin B, H and L activities during long-term storage of two weeks.
276 For the sous vide experiments, the increase in cathepsin B + L activity at 50 ˚C after 1 hour of
277 cooking suggests that cathepsin B and/or L in beef brisket may also exist in the form of a pro-

enzyme, which is activated by heat. Thus, at this temperature, with a higher cathepsin B + L activity, these enzymes are likely to be involved in proteolysis and contribute to the tenderizing effect. Cathepsin B and L were found to be more heat stable at sous vide temperatures (50˚C for 24 hours, 55˚C for 5 hours and 60˚C and 70˚C for 1 hour) compared to cathepsin H, supporting the hypothesis that cathepsin B and L remain active at typical sous vide cooking temperatures and could be involved in the tenderization process.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Riddet Institute (NZ) CoRE and Food Industry Enabling Technologies program (contract MAUX1402) for funding. We also thank Feng Ming Chian and Anneminke Buwalda (Massey University) for technical support.

References

Agarwal, S. K. 1990. Proteases cathepsins—a view. Biochemical Education, 18(2), 67-72.

Allen RE, Goll DE. 2003. Cellular and developmental biology of skeletal muscle as related to muscle growth. In Biology of Growth of Domestic Animals. Scanes CG (ed). pp 148-169. Blackwell Publishing, Iowa State Press, USA

Baldwin DE. 2012. Sous vide cooking: A review. Int J Gastr Food Sci 1: 15-30. doi:10.1016/j.ijgfs.2011.11.002

Bandman E, Zdanis, D. 1988. An immunological method to assess protein degradation in post-mortem muscle. Meat Sci 22:1-19. doi: 10.1016/0309-1740(88)90023-X

Beltrán, J., Bonnet, M., & Ouali, A. 1992. Comparative action of cathepsins B and L on intramuscular collagen as assessed by differential scanning calorimetry. Meat science, 32(3), 299-306.

Bowker BC, Eastridge JS, Paroczay EW, Callahan JA, Solomon MB. 2010. Aging/tenderization mechanisms. In Handbook of Meat Processing. Toldrá F (ed). pp 87-104. Blackwell Publishing, Iowa, USA.

Burleigh, M. C., Barrett, A. J., & Lazarus, G. S. 1974. Cathepsin B1. A lysosomal enzyme that degrades native collagen. Biochemical Journal, 137(2), 387-398.

Chéret R, Delbarre-Ladrat C, de Lamballerie-Anton M, Verrez-Bagnis V. 2007. Calpain and cathepsin activities in post mortem fish and meat muscles. Food Chem 101: 1474-1479. doi: 10.1016/j.foodchem.2006.04.023

Christensen L, Ertbjerg P, Aaslyng MD, Christensen M. 2011. Effect of prolonged heat treatment from 48 C to 63 C on toughness, cooking loss and color of pork. Meat Sci 88: 280-285. doi: 10.1016/j.meatsci.2010.12.035

Christensen, L., Ertbjerg, P., Løje, H., Risbo, J., van den Berg, F. W., & Christensen, M. 2013. Relationship between meat toughness and properties of connective tissue from cows and young bulls heat treated at low temperatures for prolonged times. Meat science, 93(4), 787-795.

Christian J, Stephen JJ. 2010. Freezing/Thawing. In Handbook of Meat Processing. Toldrá F (ed). pp 105-124. Wiley Blackwell Publishing, Iowa, USA.

Dominguez-Hernandez E, Salaseviciene A, Ertbjerg P. 2018. Low-temperature long-time cooking of meat: Eating quality and underlying mechanisms. Meat Sci 143: 104-113. doi: 10.1016/j.meatsci.2018.04.032

Ertbjerg P, Christiansen LS, Pedersen AB, Kristensen L. 2012. The effect of temperature and time on activity of calpain and lysosomal enzymes and degradation of desmin in porcine

longissimus muscle. 58th International Congress of Meat Science and Technology, Montreal, QC, Canada.

Hamm R, Deatherage FE. 1960. Changes in hydration, solubility and charges of muscle proteins during heating of meat. Journal Food Sci 25: 587-610. doi:10.1111/j.1365-2621.1960.tb00004.x

Herrera-Mendez CH, Becila S, Boudjellal A, Ouali A. 2006. Meat ageing: Reconsideration of the current concept. Trends Food Sci Technol 17: 394-405. doi: 10.1016/j.tifs.2006.01.011

Koohmaraie M, Geesink G. 2006. Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. Meat Sci 74: 34-43. doi: 10.1016/j.meatsci.2006.04.025

Koohmaraie M, Seideman S, Schollmeyer J, Dutson T, Babiker A. 1988. Factors associated with the tenderness of three bovine muscles. J Food Sci 53: 407-410. doi: 10.1111/j.1365-2621.1988.tb07717.x

Laakkonen E, Wellington G, Sherbon J. 1970. Low-temperature, long-time heating of bovine muscle 1. Changes in tenderness, water-binding capacity, pH and amount of water-soluble components. J Food Sci 35: 175-177. doi: 10.1111/j.1365-2621.1970.tb12131.x

Lana A, Zolla L. 2016. Proteolysis in meat tenderization from the point of view of each single protein: A proteomic perspective. J Proteom 147: 85-97.

Matos EAD. 2013. Nutrition and farming practices as modulators of quality in gilthead seabream (Sparus aurata) Ph. D. thesis, Univ. Algarve, Portual.

Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez CH, Sentandreu MA. 2013. Biomarkers of meat tenderness: present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat Sci 95: 854-870. doi: 10.1016/j.meatsci.2013.05.010

Pomponio L, Ertbjerg P. 2012. The effect of temperature on the activity of μ-and m-calpain and calpastatin during post-mortem storage of porcine longissimus muscle. Meat Sci 91: 50-55. doi: 10.1016/j.meatsci.2011.12.005

Rico E, Toldrá F, Flores J. 1991a. Assay of cathepsin D activity in fresh pork muscle and dry-cured ham. Meat Sci 29: 287-293.

Rico E, Toldrá F, Flores J. 1991b. Problems associated with the assay of cathepsin D in meat and meat products. Food Chem 40: 87-91.

Solvig H. 2014. The effect of proteolytic lysosomal enzymes and cathepsin B on collagen and connective tissue denaturation during long time low temperature cooking. Masters thesis, Stavanger University, Norway.

Spanier A, McMillin K, Miller J. 1990. Enzyme activity levels in beef: Effect of postmortem aging and end-point cooking temperature. J Food Sci 55: 318-322.

Toldrá F, Etherington DJ. (1988). Examination of cathepsins B, D, H and L activities in dry-cured hams. Meat Sci 23: 1-7.

van Jaarsveld FP, Naudé RJ, Oelofsen W. 1997. The effects of Ca ions, EGTA and storage time on myofibrillar protein degradation, levels of Ca2+-dependent proteases and cathepsins B, H, L and D of ostrich skeletal muscle. Meat Sci 45: 517-529.

Zhu X, Kaur L, Staincliffe M, Boland M. (2018). Actinidin pretreatment and sous vide cooking of beef brisket: Effects on meat microstructure, texture and in vitro protein digestibility. Meat Sci 145: 256-265.

375 **Figure Legends**

376 Figure 1. Experimental plan.

377 Figure 2. Relative activity of cathepsin B + L in hot boned beef brisket sous vide cooked at
378 50, 55, 60, 65 and 70 ˚C for 1, 5 and 24 hours.

379 Figure 3. Relative activity of cathepsin B in hot boned beef brisket sous vide cooked at 50,
380 55, 60, 65 and 70 ˚C for 1, 5 and 24 hours.

381 Figure 4. Relative activity of cathepsin L in hot boned beef brisket sous vide cooked at 50,
382 55, 60, 65 and 70 ˚C for 1, 5 and 24 hours.

383

384

385

386

387

388

389

390

Table 1. pH and activities of endogenous enzymes (cathepsin B and H) in 6 hours *post-mortem* hot boned beef brisket (control) and subsequent storage at either 4 ˚C or -20 ˚C for 4 days

	6 hours *post-mortem* meat (Control)	4 days *post-mortem* meat stored at 4 ˚C	4 days *post-mortem* meat stored at -20 ˚C
pH	5.78 ± 0.07^{A}	5.67 ± 0.04^{A}	5.84 ± 0.05^{A}
Cathepsin B[1]	13004 ± 2837^{C}	36965 ± 3294^{A}	27748 ± 2331^{AB}
Cathepsin H[1]	25066 ± 4508^{A}	16910 ± 4652^{A}	14770 ± 1335^{A}

Different letters in each row are significantly different ($P < 0.05$).

All values are mean ± standard error of mean for three replicates.

[1]The units for the enzyme activities are expressed as increase in FU per min per g of muscle for cathepsin activities.

Table 2. pH and activities of endogenous enzymes (cathepsin B and H) in 4 days *post-mortem* hot boned beef brisket stored at 4 ˚C (control) and subsequent storage at either 4 ˚C or -20 ˚C for 14 days

		Storage at 4 ˚C		Storage at -20 ˚C	
	4 days *post-mortem* meat stored at 4 ˚C (Control)	+ 7 days *post-mortem* meat	+ 14 days *post-mortem* meat	+ 7 days *post-mortem* meat	+ 14 days *post-mortem* meat
pH	5.67 ± 0.04^{A}	5.65 ± 0.06^{A}	5.51 ± 0.02^{A}	5.60 ± 0.12^{A}	5.55 ± 0.13^{A}
Cathepsin B[1]	36965 ± 3294^{A}	50458 ± 16464^{A}	63613 ± 8650^{A}	48666 ± 16037^{A}	48116 ± 23234^{A}
Cathepsin B+L[1]	n.d.	551166 ± 163651^{A}	737351 ± 57530^{A}	602593 ± 82243^{A}	532009 ± 149737^{A}
Cathepsin L[1]	n.d.	500708 ± 147721^{A}	673738 ± 63454^{A}	553927 ± 72387^{A}	483892 ± 131116^{A,}
Cathepsin H[1]	16910 ± 4652^{A}	36950 ± 28727^{A}	17793 ± 2549^{A}	16558 ± 11158^{A}	16469 ± 9480^{A}

Different letters in each row are significantly different ($P < 0.05$).

All values are mean ± standard error of mean for three replicates.

[1]The units for the enzyme activities are expressed as increase in FU per min per g of muscle for cathepsin activities.

n.d. Not determined

Table 3. Effect of sous vide cooking temperatures (50 to 70 ˚C) and times (0, 1, 5, 24 hours) on the pH of 1 day *post-mortem* hot boned beef brisket.

Cooking time	50˚C	55˚C	60˚C	65˚C	70˚C
0 hour	$5.84 \pm 0.05^{A, a}$	$5.84 \pm 0.05^{A, a}$	$5.84 \pm 0.05^{A, a}$	$5.84 \pm 0.05^{A, a}$	$5.84 \pm 0.05^{A, a}$
1 hour	$5.83 \pm 0.04^{B, a}$	$5.95 \pm 0.00^{A, a}$	$5.93 \pm 0.03^{AB, a}$	$5.94 \pm 0.03^{AB, a}$	$6.00 \pm 0.02^{A, a}$
5 hours	$5.78 \pm 0.02^{B, a}$	$5.88 \pm 0.03^{AB, a}$	$5.97 \pm 0.06^{A, a}$	$5.97 \pm 0.04^{A, a}$	$6.02 \pm 0.03^{A, a}$
24 hours	$5.88 \pm 0.05^{A, a}$	$5.94 \pm 0.04^{A, a}$	$5.95 \pm 0.06^{A, a}$	$5.96 \pm 0.04^{A, a}$	$6.00 \pm 0.06^{A, a}$

Different uppercase letters in each row are significantly different among cooking temperatures at the same cooking time ($P < 0.05$).

Different lowercase letters in each column are significantly different among cooking times at the same cooking temperature ($P < 0.05$).

All values are mean ± standard error of mean for three replicates.

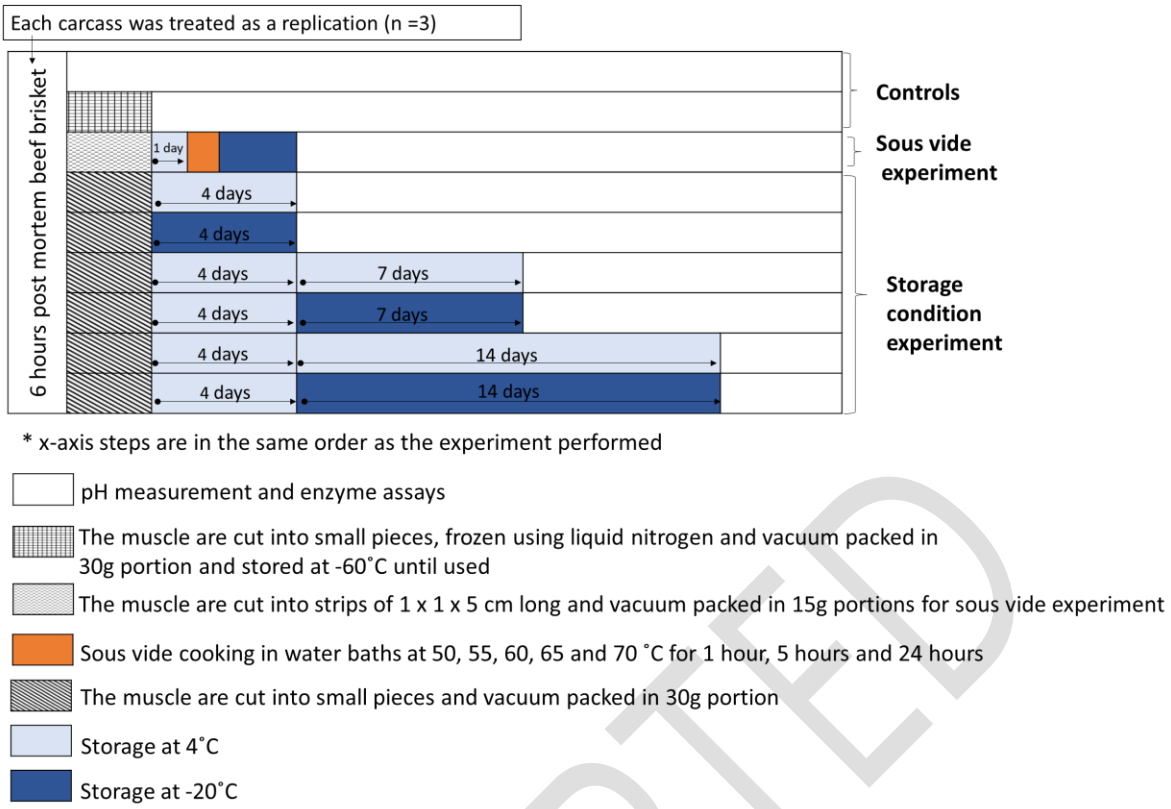
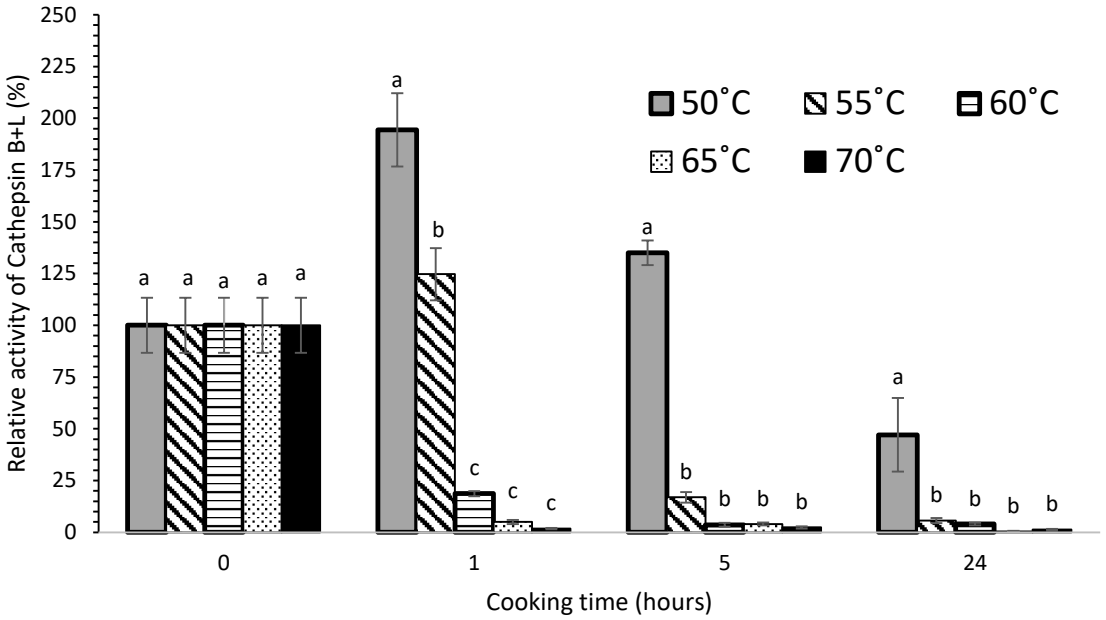


Figure 1. Experimental plan.



449

450 **Figure 2. Relative activity of cathepsin B + L in hot boned beef brisket sous vide cooked**

451 **at 50, 55, 60, 65 and 70 ˚C for 1, 5 and 24 hours.**

452 * Each data point represents the mean value from three animals (error bars indicate SE).

453 Different lowercase letters are significantly different among cooking temperatures at the

454 same cooking time ($P < 0.05$).

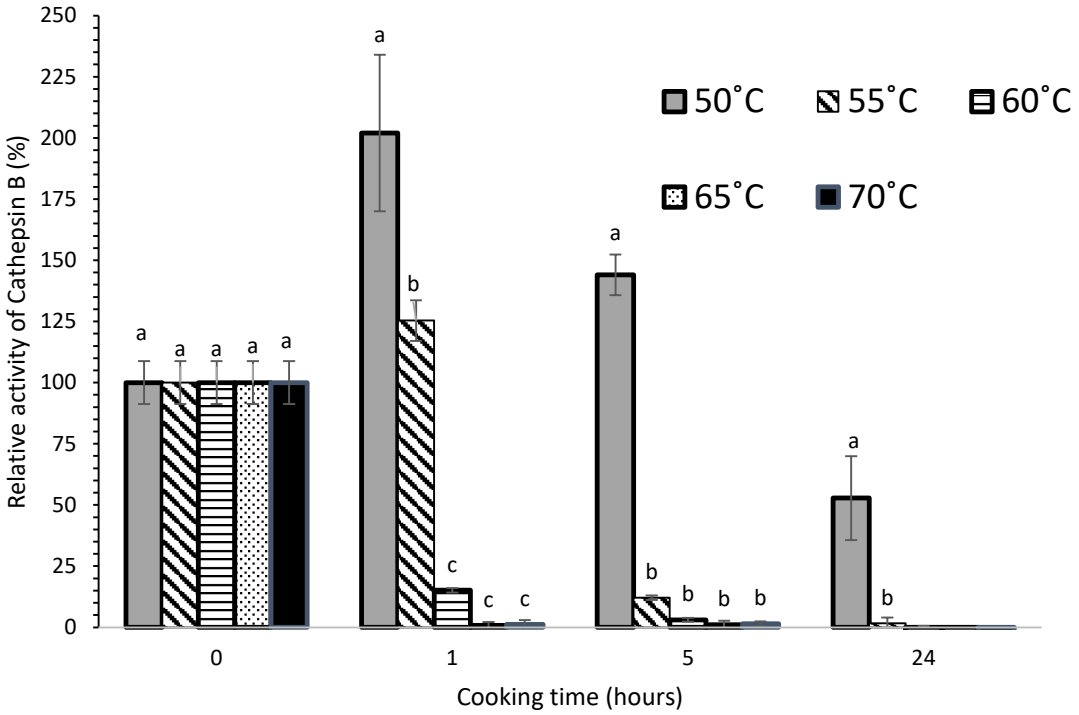
455

456

457

458

459



460

461 **Figure 3. Relative activity of cathepsin B in hot boned beef brisket sous vide cooked at**

462 **50, 55, 60, 65 and 70 ˚C for 1, 5 and 24 hours.**

463 * Each data point represents the mean value from three animals (error bars indicate SE).

464 Different lowercase letters are significantly different among cooking temperatures at the

465 same cooking time ($P < 0.05$).

466

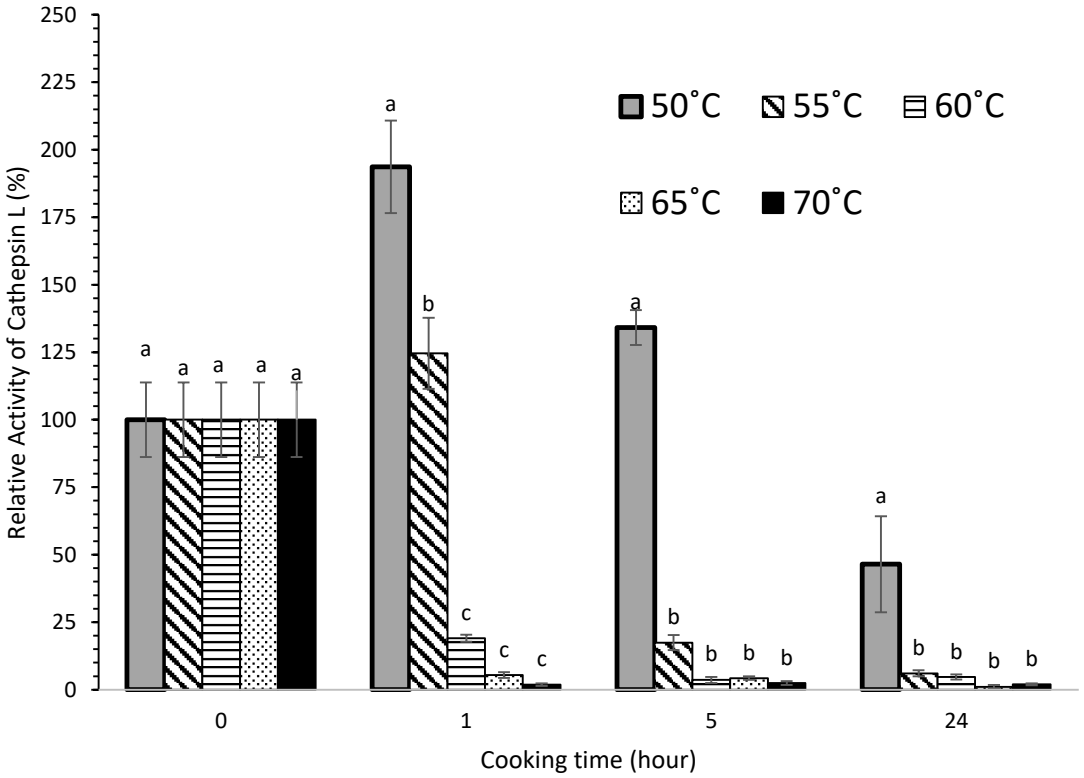


Figure 4. Relative activity of cathepsin L in hot boned beef brisket sous vide cooked at 50, 55, 60, 65 and 70 ˚C for 1, 5 and 24 hours.

* Each data point represents the mean value from three animals (error bars indicate SE).

Different lowercase letters are significantly different among cooking temperatures at the same cooking time ($P < 0.05$).