SOUS VIDE PROCESSING TO TENDERISE MEAT

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ABSTRACT

The development of meat tenderness during *sous vide* cooking is associated with the proteolysis of meat proteins by endogenous enzymes, such as cathepsins B and L, and collagen solubilisation. However, these two processes are optimal at different temperature regimes. The aim of this thesis was to evaluate the effect of combining both temperature regimes through two-stage *sous vide* cooking on meat tenderness and cooking loss, as well as its ability to reduce total cooking time compared to conventional single-temperature *sous vide* cooking. This thesis also aimed to determine the roles of cathepsin B and L, collagen denaturation, and collagen solubilisation in the development of meat tenderness. Data for this thesis were obtained using beef *pectoralis profundus* (brisket), which is a tough meat cut that has the potential to gain tenderness through *sous vide* cooking.

In this thesis, the kinetics of cathepsin B and L activity during *sous vide* cooking of beef brisket were evaluated at the temperatures of 46 – 60 °C for up to 72 h. Their activity initially increased, then decreased with longer cooking time. These changes in cathepsin B and L activity were successfully modeled using consecutive reactions in series, which are activation (the release of cathepsin from lysosomes) followed by deactivation (thermal denaturation). The model allows for the calculation of the cumulative activity of cathepsins for a given temperature and time. It was found that a greater cumulative activity can be achieved by *sous vide* cooking at lower temperatures such as 46 °C for a prolonged time compared to a higher temperature such as 56 °C.

To evaluate the effect of precooking at different cumulative cathepsin B and L activities on meat tenderness, cooking loss, and collagen solubilisation, beef brisket was precooked at the temperatures of 46 °C and 56 °C for up to 72 h, followed by cooking at 71 °C. No change in the shear force value of meat precooked at 46 °C was observed, while a decrease in the shear force value was noted in meat precooked at 56 °C with increasing cooking time. Meat precooked at 56 °C also showed higher collagen solubilisation than meat precooked at 46 °C, while no difference in cooking loss was observed between the two precooking temperatures. From this work it was

concluded that higher cumulative cathepsin B and L activity did not result in tenderisation of meat indicating that cathepsin B and L do not contribute to the development of tenderness in tough meat cuts like brisket. On the other hand, meat tenderness appears to be correlated with collagen solubilisation. A further study using precooking at different cumulative activities (46 – 56 °C for 0 – 12 h), followed by cooking at 80 °C for 8 h, supports the result that cathepsins B and L do not contribute to meat tenderisation during *sous vide* cooking. In this study, beef brisket precooked at 46, 50, and 53 °C for 12 h and at 56 °C for 6 and 12 h had a lower shear force value than meat cooked directly at 80 °C for 8 h. Faster tenderisation was observed as early as 6 h with precooking at 56 °C. *Sous vide* cooking at 56 °C for 6 and 12 h was characterised by lower cumulative cathepsin B and L activity, a higher degree of collagen denaturation, and a marked decrease in final yield force compared to other temperature-time combinations.

The kinetics of collagen denaturation in *sous vide*-cooked beef brisket were also determined in this thesis, using the enthalpy of denaturation (ΔH) of the second peak (Peak 2) from the thermogram generated by differential scanning calorimetry (DSC). The change in the ΔH of Peak 2 was successfully modeled using a three-protein model. The simulation of collagen denaturation using kinetic parameters obtained from the model showed a marked increase in collagen denaturation at 56 °C.

A further study using precooking at 56 °C for 6 h, followed by cooking at various second-stage temperature-time combinations (60, 70, 80, and 90 °C for 0 – 20 h), showed that two-stage *sous vide* cooking could not reduce the total cooking time to tenderise meat compared to conventional single-temperature *sous vide* cooking. Based on conventional single-temperature *sous vide* cooking, the time required for beef brisket to reach the shear force correlated with the consumer tenderness threshold (Liang et al., 2016) was determined. It was found that at 60, 70, 80, and 90 °C, the times required to achieve the consumer tenderness threshold were 20, 26, 12, and 8 h, respectively. Although shorter times were needed to achieve the consumer tenderness threshold at 80 and 90 °C, these temperatures resulted in significantly higher cooking loss compared to 60 °C.

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In memory of two incredible people,

my mother and grandmother,

To my mom, Siti Habedah Abdullah, who encouraged me to pursue my doctoral degree, even though you knew it would mean being far from you for quite a long time. Thank you for your love and for always believing in me. I will always cherish our memories together, especially the time we spent traveling in New Zealand. I believe you are happy knowing that I have completed my doctoral degree.

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AMC	7-amino-4-methylcoumarin
Ca2Cl	calcium chloride
CL	cook loss fraction
ColCL	solubilised collagen in cook loss
ColMF	collagen in meat residue
DF	Dilution factor
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HMM-S2	Heavy meromyosin subfragment-2
HMM S-1	Heavy meromyosin subfragment-1
LMM	Light meromyosin
MR	Meat residue fraction
NaCl	Sodium chloride
PPII	Polyproline II
RCS	Refrigerating cooling system
RFU	Relative fluorescence unit
SC	Solubilised collagen in cook loss per total collagen
sHSP	Small heat shock proteins
SEM	Standard error of the mean

SP	Sarcoplasmic proteins
Tc	Core temperature of the steak reached the temperature of the water bath
TPP	Sodium tripolyphosphate
Z-Phe-Arg-AMC	Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride
Z-RR-AMC	Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride

CHAPTER 1 Introduction

The red meat is New Zealand's second largest industry, contributing up to 16 % to the country's export revenue in 2021–22 with the total value of $11 billion, in which beef accounts for $4.6 billion (Meat Industry Association, 2022). Nevertheless, within the meat sector, the value of meat varies across different muscle cuts. Meat derived from locomotive muscles, such as brisket, shank, eye of round is inherently tough, leading to reduced consumer acceptance. Consequently, these tougher cuts are marketed at lower prices in comparison to more tender cuts such as tenderloin and striploin (Jeremiah, Gibson, Aalhus, & Dugan, 2003; Pfeiffer, Voges, King, Griffin, & Savell, 2005). In order to increase the value of these tougher cuts, the meat industry has been exploring *sous vide* processing as a method to tenderise these cuts (Fareh, 2018; Thathsarani, Alahakoon, & Liyanage, 2022).

Tenderness and juiciness are important meat quality attributes that determine consumer eating satisfaction (Becker, Boulaaba, Pingen, Krischek, & Klein, 2016). Beyond these meat intrinsic attributes, consumer preference for meat is influenced by convenience in preparation and price. The reduction in consumer consumption of red meat among other factors is attributed to the demand for quick meal preparation and the high price of red meat (Realini et al., 2023; Resurreccion, 2004). The use of *sous vide* processing to tenderise low-value meat cuts provides an opportunity for the meat industry to meet consumer demands for convenient, ready-to-eat tender meat at affordable prices. However, this processing method still presents a considerable challenge to the meat industry mainly due to the long processing time, making it economically impractical. Typically, tough meat cuts are *sous vide* cooked at temperature/time ranges of 55 – 60 °C for 24 to 48 h, or 80 – 85 °C for 12 to 16 h (Baldwin, 2021). The financial feasibility analysis has demonstrated that a reduction in cooking time could lead to a more cost-effective *sous vide* processing operation, providing the meat industry a profitable method for tenderisation of tough meat cuts (Fareh, 2018).

Sous vide is a cooking method that involves cooking foods inside heat-stable vacuum-sealed pouches under precise, controlled conditions of temperature and time (Baldwin, 2012). The tenderisation that occurs during *sous vide* cooking is associated with proteolysis of myofibrillar and connective tissue proteins by meat endogenous enzymes, such as cathepsin B and L, and collagen solubilisation (Christensen et al., 2013; Dominguez-Hernandez, Salaseviciene, & Ertbjerg, 2018). However, these two processes are optimal at different temperature regimes. Increasing cooking temperature from 50 to 90 °C causes an increase in the rate of cathepsin B and L deactivation and collagen solubilisation (Fareh, 2018; Kaur, Hui, & Boland, 2020). A different approach to *sous vide* cooking meat that can combine the benefits of both temperature regimes might provide possibilities for reducing total cooking time, as well as improving meat tenderness and juiciness. This may be achieved using two-stage sous vide, which involves initial low-temperature *sous vide* (first stage) followed by high-temperature *sous vide* (second stage) to promote collagen solubilisation. It is hypothesised that two-stage *sous vide* cooking can act on two mechanisms: meat protein proteolysis by endogenous enzymes, such as cathepsins B and L, during the low-temperature *sous vide* cooking (first stage) and rapid collagen solubilisation during the high-temperature *sous vide* cooking (second stage). Thus, combining the two processes may provide a more rapid tenderisation. Consequently, the aim of this thesis was to investigate the effect of two-stage *sous vide* cooking on meat tenderness and cooking loss, and its ability to reduce total cooking time compared to conventional single-temperature *sous vide* cooking. This thesis also aimed to determine the roles of cathepsin B and L, collagen denaturation, and collagen solubilisation in the development of meat tenderness during *sous vide* cooking. Beef brisket (*pectoralis profundus*) is chosen for this study because it is an under-valued and less preferred cut of beef, primarily due to its tough characteristics (Kukowski, Maddock, & Wulf, 2004; Oh et al., 2016). However, it has the potential to gain value through *sous vide* cooking. The findings obtained would provide a better understanding of the underlying mechanism of meat tenderisation during two-stage *sous vide* cooking and enable the meat industry to customize the *sous vide* processing conditions to meet the meat quality and time requirements.

Thesis structure

Chapter 1 describes the background and outlines the main framework of the research project.

Chapter 2 provides a literature review on the background of *sous vide* cooking, meat structure and composition, the impact of *sous vide* cooking on tenderness and juiciness, cathepsin B and L, and the two-stage *sous vide* cooking.

Chapter 3 describes the kinetic model to predict cathepsin B and L activity during *sous vide* cooking and the effect of temperature on their rate of reaction.

Chapter 4 compares the effects of cooking at different levels of cumulative cathepsin B and L activity on protein thermal denaturation profile, meat texture, cooking loss, and collagen solubility.

Chapter 5 determines the kinetic model to predict collagen thermal denaturation in beef brisket.

Chapter 6 investigates the effect of two-stage *sous vide* cooking compared to single-stage *sous vide* cooking on meat texture, cooking loss, and collagen solubility at two conditions: 1) different temperature-time combinations of the first stage and fixed second stage and 2) fixed first stage and different temperature-time combinations of the second stage.

Chapter 7 provides a discussion of all research findings and suggests directions for future work.

CHAPTER 2 Literature review

2.1 *Sous vide*

Sous vide is a French term that means 'under vacuum'. It is a cooking technique that involves placing food in a heat-stable pouch, sealing it under vacuum, and heating it in a temperature-controlled water bath at low temperatures (< 100 °C) for extended periods of time (Aguilera, 2018). It was invented back in 1970 – 1974 in France by Bruno Goussault and Georges Pralus, who worked independently to cook roast beef and foie gras, respectively, with reduced cooking loss (Hesser, 2005). This cooking technique then became popular among chefs and is widely used in commercial kitchens and restaurants. It is also being adopted in home kitchens using water baths with portable clip-on immersion circulators (Aguilera, 2018).

The low heating temperatures bring about a slow rise in meat temperature during *sous vide* cooking, thus giving benefits in terms of producing meat with uniform doneness from the edges to the center (Bejerholm, Tørngren, & Aaslyng, 2014b) (Figure 2.1). Additionally, the low heating temperature applied in *sous vide* cooking produces meat with more desirable tenderness and juiciness compared to conventional cooking methods that use higher heating temperatures, such as oven roasting (160 – 200 °C) (James & Yang, 2012; Mitra, Rinnan, & Ruiz-Carrascal, 2017). Besides, the application of vacuum packaging before cooking produces meat products with a longer shelf life due to the elimination of the risk of recontamination after cooking and during storage (Baldwin, 2012). This process also inhibits the growth of aerobic bacteria and prevents lipid oxidation due to the oxygen-deficient environment within the packaging (Church & Parsons, 1993). Furthermore, vacuum packaging not only removes headspace inside the packaging, allowing for efficient heat transfer during cooking, but it also seals in volatile flavour compounds within the package and prevents the evaporative loss of water during cooking (Baldwin, 2012; Bejerholm, Tørngren, & Aaslyng, 2014a; Rinaldi et al., 2014).

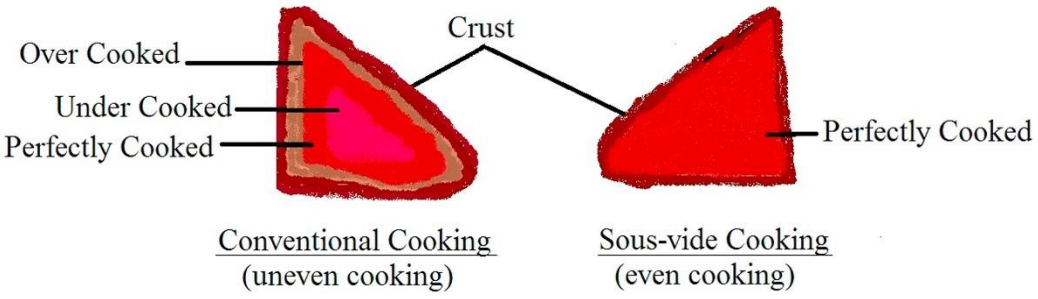

Figure 2. 1 Conventional cooking in comparison with *sous vide* cooking (Ayub & Ahmad, 2019).

Sous vide cooking is generally performed using a water bath or a convection steam oven. While convection steam ovens can accommodate a large quantity of food, they often have a non-uniform heat distribution (Baldwin, 2012). In contrast, circulating water baths offer a uniform heat distribution and typically maintain temperature swings of less than 0.1 °C (Baldwin, 2012). Following the heating process, the products need to be cooled, and the chilling process should be rapid to minimise the time spent in the temperature danger zone (5 – 60 °C), which could promote the germination of any surviving bacterial spores (Coorey et al., 2018). This cooling process can be achieved using air blast chillers, water immersion, or conduction chillers. (Church & Parsons, 1993).

The basis of *sous vide* cooking is to cook meat at a temperature below the point at which muscle fibres shrink, but long enough to make collagen solubilise, thus producing meat with extraordinary tenderness and juiciness, especially for low-temperature *sous vide* cooking (<65 ˚C) (Keller, 2008). Prominent chefs, such as Bruno Goussault, suggest *sous vide* cooking tough meat cuts at 60°C for 24 – 48 h or at 83 °C for 12 – 15 h ("*Sous-vide* The Art & Science of *Sous-vide* Cooking," 2024). Thomas Keller suggests *sous vide* cooking tender cuts of meat at 59.5 – 60.5 °C and tough cuts of meat at 74 – 80 ˚C (Keller, 2008). Baldwin (2021) suggests *sous vide* cooking tough cuts of meat at 55 – 60 °C and holding for a long time or at 80 °C and holding for a shorter time (Table 2.1).

Table 2. 1 *Sous vide* culinary suggestion.

Meat cut		Temperature (°C)	Time	References
Tough cut	Brisket	60	24 h	("*Sous-vide* The Art & Science of *Sous-vide* Cooking," 2024)
	Short rib	60	48 h	
	Beef cheeks	83	12 h	
	Lamb shank	83	15 h	
Tender cut	Tenderloin	56	1 h	
Tough cut	Pork leg and shoulder	80	8 h	Keller (2008)
	Rabbit flanks	74	12 h	
Tender cut	Beef sirloin	59.5	45 min	
	Veal tenderloin	61	30 min	
	Lamb saddle	60.5	35 min	
	Pork rack and saddle	60.5	20 min	
Tough cut		55 – 60	24 – 48 h	Baldwin (2021)
		80 – 85	12 – 16 h	

2.2 Meat structure and composition

Skeletal muscle mainly consists of muscle fibres and intramuscular connective tissue. Intramuscular connective tissue is distributed on three levels of scale in the muscle: the endomysium, which surrounds each muscle fibre, the perimysium, which compartmentalizes muscle in fibre bundles, and finally the epimysium, which is the external envelope of muscle (Figure 2.2) (Listrat et al., 2016). Larger (primary) muscle bundles may consist of a number of smaller (secondary) muscle bundles, separated by thinner layers of perimysium (Purslow, 2017).

Meat composes approximately 75 % water, 19 % protein, 2.5 % lipid, 1.2 % carbohydrate, and 2.5 % miscellaneous soluble nonprotein substances and vitamins (Table 2.2) (López-Bote, 2017). Meat proteins consist of 50 – 55 % myofibrillar proteins, 30 – 34 % sarcoplasmic proteins, and 10 – 15 % connective tissue proteins (Tornberg, 2005). Sarcoplasmic proteins exist in sarcoplasm and consist of heme pigments (myoglobin), glycolytic enzymes, mitochondria, oxidative enzymes, the nucleus, lysosomes, and other components (Boland, Kaur, Chian, & Astruc, 2019).

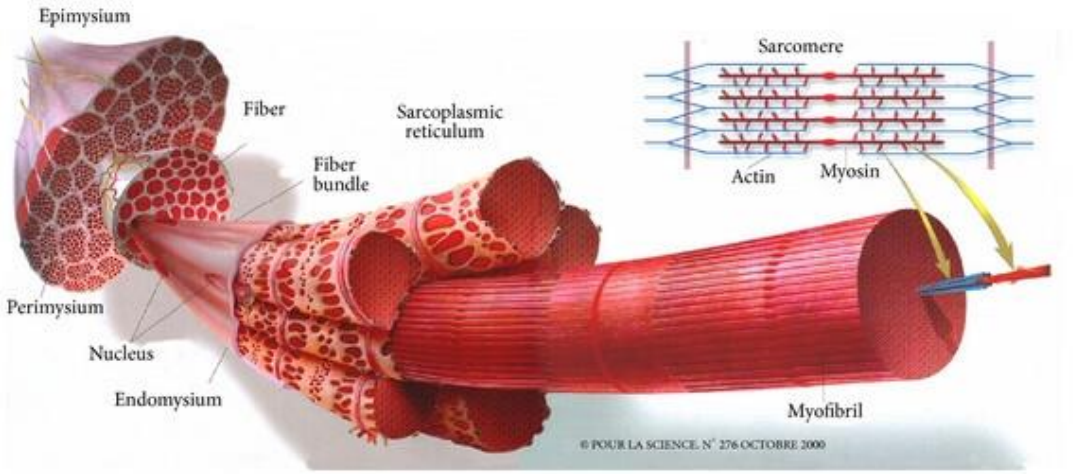

Figure 2. 2 General organization of the muscle (Listrat et al., 2016).

Table 2. 2 Meat composition (López-Bote, 2017).

	Components		Composition (%)
1	**WATER**		75
2	**PROTEIN**		19
	(a) Myofibrillar protein	11.5	
	Myosin[a] (H- and L-meromyosins and several light chain components associated with them)	5.5	
	Actin[a]	2.5	
	Connectin (titin)	0.9	
	N2 line protein (nebulin)	0.3	
	Tropomyosins	0.6	
	Troponins, C, I and T	0.6	
	α, β and γ actinins	0.5	
	Myomesin, (M-line protein) and C-proteins	0.2	
	Desmin, filamin, F- and I-proteins, vinculin, talin, etc.	0.4	
	(b) Sarcoplasmic protein	5.5	
	Glyceraldehyde phosphate dehydrogenase	1.2	
	Aldolase	0.6	
	Creatine kinase	0.5	
	Other glycolytic enzymes especially phosphorylase	2.2	
	Myoglobin	0.2	
	Hemoglobin and other unspecified extracellular proteins	0.6	
	(c) Intramuscular connective tissue and organelle	2	
	Collagen	1	

	Elastin	0.05		
	Mitochondrial, etc. (including cytochrome c and insoluble enzymes)	0.95		
3	**LIPID**			2.5
	Neutral lipid, phospholipids, fatty acids, fat-soluble substances		2.5	
4	**CARBOHYDRATE**			1.2
	Lactic acid	0.9		
	Glucose-6-phosphate	0.15		
	Glycogen	0.1		
	Glucose, traces of other glycolytic intermediates	0.05		
5	**MISCELLANEOUS SOLUBLE NONPROTEIN SUBSTANCES**			2.3
	(a) Nitrogenous		1.65	
	Creatinine	0.55		
	Inosine monophosphate	0.3		
	Di- and tri-phosphopyridine nucleotides	0.1		
	Amino acids	0.35		
	Carnosine, anserine	0.35		
	(b) Inorganic		0.65	
	Total soluble phosphorus	0.2		
	Potassium	0.35		
	Sodium	0.05		
	Magnesium	0.02		
	Calcium, zinc, trace metals	0.03		
6	**VITAMINS**			
	Various fat- and water-soluble vitamins, quantitatively minute.			

[a]Actin and myosin are combined, as actomyosin in post rigor muscle (López-Bote, 2017)

2.2.1 Intramuscular connective tissue

Intramuscular connective tissue is a soft connective tissue that found in the body of any muscle but do not include tendon (Bailey & Light, 1989). Intramuscular connective tissue consists of extracellular matrix components mainly collagen and relatively small amount of elastin and proteoglycans (Purslow, 2010). The structure of Intramuscular connective tissue is divided into three levels of organization (endomysium, perimysium, and epimysium) Figure 2.2. It is important to note that overlying sarcolemma (muscle fibre plasma membrane) before the endomysium is an extracellular membrane component known as basement membrane (40–50 nm thickness) that is composed of 40 % non-fibrillar collagen (collagen type IV), 1 – 5 %

proteoglycan (heparan sulphate), and glycoprotein (laminin, nidogen, and fibronectin) (Bailey & Light, 1989).

The epimysium normally removed during meat preparation thus does not contribute to the variation in cooked meat tenderness. The endomysium is composed of a dense and random network of collagen fibrils 30 – 70nm in diameter (Figure 2.3b and 2.3d). The perimysium is composed of several layers of 100 – 200µm-thick sheets of collagen fibres (Figure 2.3a) that has a regular wavy pattern (Figure 2.3c). Some collagen fibrils form a loose network at the surface of the perimysial sheet that attaches to the collagen fibres in adjacent perimysium sheets (Figure 2.3c) (Nishimura, Hattori, & Takahashi, 1994). In terms of arrangement, collagen fibres are organized into two layers, with the collagen fibres in each layer arranged parallel to one another. In resting muscle, these layers are oriented at an angle of approximately ± 54° relative to the direction of the muscle fibres (Figure 2.4) (Purslow, 2005).

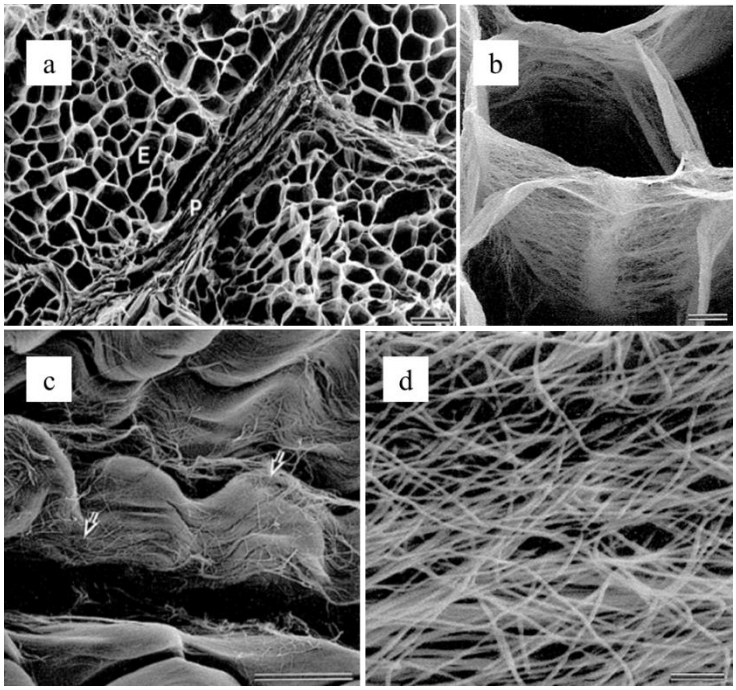

Figure 2. 3 a. Endomysium (E) and perimysium (P), x150. Bar = 100µm. b. Endomysial sheath, x1100. Bar = 10µm. c. Perimysium, bundles of collagen fibres in a wavy pattern covered with loose network of collagen fibrils (arrow), x2000. Bar = 10µm. d. The random arrangement of collagen fibrils in the endomysium, x21500. Bar = 500nm (Nishimura et al., 1994).

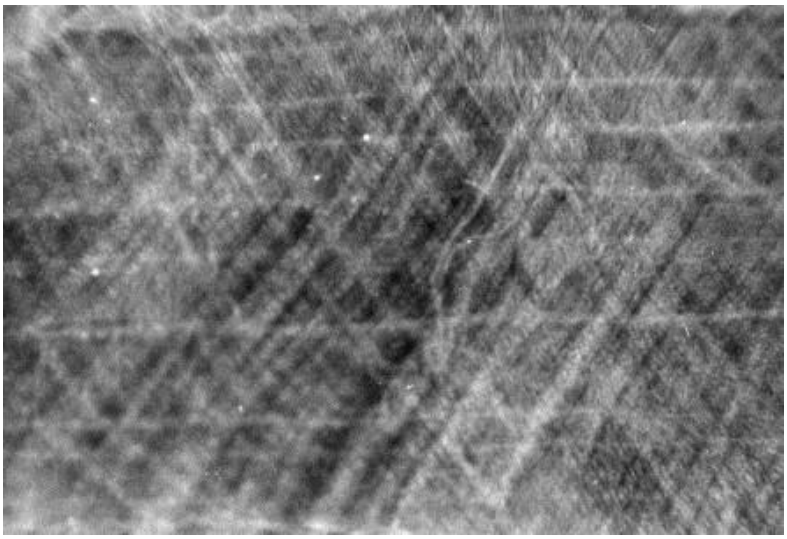

Figure 2. 4 Light micrograph of the epimysium from the bovine *sternomandibularis* muscle, showing the arrangement of collagen fibres (Purslow, 2005).

Collagen is the main protein in the intramuscular connective tissue. In vertebrates and higher invertebrates, there are 28 distinct collagen types. These collagens have been given Roman numeral designations (I–XXVIII) in chronological order of discovery and they are classified by type based on both their domain structure and their suprastructural organization (Mienaltowski & Birk, 2014). In intramuscular connective tissue, seven types of collagens are present. The epimysium contains collagen type I as a major component and collagen type III as a minor component. Similarly, the perimysium contains collagen type I as a major component and collagen type III as a minor component. Additionally, the perimysium also contains collagen types V, VI, XII, and XIV. In contrast, the endomysium contains collagen types I and III as major components, while collagen types V, IV, and VI are present as minor components (Bailey, Restall, Sims, & Duance, 1979; Listrat et al., 2000; Nishimura, Ojima, Hattori, & Takahashi, 1997).

The common structural feature of collagens is the presence of three polypeptides (α chains) that form a triple helix. In fibril-forming collagen, the three α chains are three left-handed polyproline II (PPII) helices intertwine to form a right-handed triple helix with a one-residue stagger between adjacent α chains. Each α chain has repeating Xaa-Yaa-Gly sequence. The presence of glycine in

every 3 amino acids residue along the α chain is important for the close packing of collagen triple helix where glycine is oriented into the centre of the helix while X and Y residues are exposed to the surface (Bailey & Light, 1989). Xaa and Yaa can be any amino acids, but mostly proline (28 %) and hydroxyproline (38 %), respectively (Shoulders & Raines, 2009).

Fibril-forming collagens are synthesized as procollagen molecules. The molecule consists of an N-terminal propeptide, a short non-helical domain at N-terminal (N-telopeptide), a central triple helical domain, a short non-helical domain at C-terminal (C-telopeptide) and a C-terminal propeptide (Figure 2.5). Before they are assembled to form fibrils, both N-propeptide and C-propeptide of procollagen are cleaved by specific procollagenase enzymes, leaving a short non-helical domain at N-terminal (N-telopeptide), a central triple helical domain, a short non-helical domain at C-terminal (C-telopeptide) as collagen (Ricard-Blum, 2011).

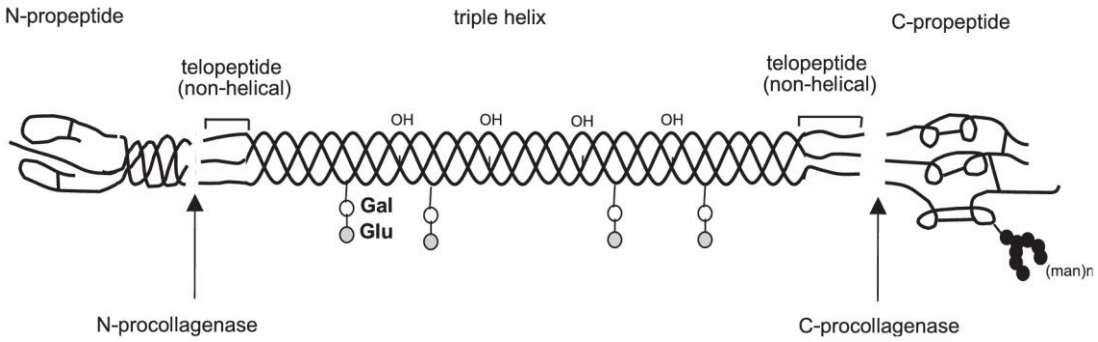

Figure 2. 5 Molecular structure of type I collagen molecule with the various subdomains as well as the cleavage sites for N- and C-procollagenases (Gelse, Pöschl, & Aigner, 2003).

The collagen triple helix is stabilized by interchain hydrogen bonds between amide group (N−H) of the glycine (Gly) and the carbonyl group (C=O) of the Xaa residue in an adjacent strand (Brodsky & Ramshaw, 1997). Total proline and hydroxyproline content also contribute to triple helix stabilization. Hydroxyproline is derived from the post-translational hydroxylation of proline (Shoulders & Raines, 2009). It is suggested that the mechanisms of triple-helix stabilization by hydroxyproline are due to the formation of water-mediated hydrogen bonds and a stereoelectronic

effect (Shoulders & Raines, 2009). This is supported by species dependent increase of hydroxyproline content correlated to an increase collagen thermal stability in solution. For example, cod that live at very low temperature contain skin collagen with reduced quantity of hydroxyproline since lower thermal melting point is important for its metabolism (Bailey & Light, 1989).

Collagen monomers are thermally labile and denatured at temperature 37 °C (Leikina, Mertts, Kuznetsova, & Leikin, 2002). In intramuscular connective tissue, collagen self-assemble to form collagen fibres, which increases their mechanical strength and raises the thermal denaturation temperature from 37 °C to 60 °C (Torrescano, Sánchez-Escalante, Giménez, Roncalés, & Beltrán, 2003). Collagen fibres are then further stabilized by the formation of reducible cross-links, which is further replaced by mature thermal stable cross-link as animal maturity increase (McCormick, 1999).

Elastin is an insoluble polymer of the monomeric soluble precursor tropoelastin and the main component of elastic fibres in matrix tissue (Halper & Kjaer, 2014). Most bovine muscles contain less than 0.2 % elastin on the dry weight basis and less than 5 % of the total collagen content (Bendall, 1967). However, some muscle such as *semitendinosus*, *latissimus dorsi*, and *penniculus* show significantly high proportion of elastin, with 1.82, 2.0, and 1.13 % elastin on the dry weight basis and 37, 33, and 10.4 % of the total collagen content, respectively (Bendall, 1967). In contrast, other muscles, such as the *pectoralis superficialis*, contain intermediate amounts of elastin, with 0.41 % elastin on a dry weight basis and 5.5 % of the total collagen content (Bendall, 1967).

2.2.2 Myofibrillar proteins

Myofibrillar proteins consist of contractile, regulatory, and structural proteins. The myofibrillar contractile proteins are composed of myosin and actin, which form the thin and thick filaments, respectively, and are involved in the skeletal muscle contraction and relaxation (Boland et al., 2019). Thin and thick filaments interdigitate and are precisely arranged in a hexagonal array. Their

precise arrangement and different optical properties produce the striated appearance of the longitudinal section of muscle fibres seen under the light microscope that composed of Z-line, A, band, H-zone, and I-band (Figure 2.6) (Purslow, 2017). Sarcomere is the smallest contractile unit of muscle and described as the region from one Z-line to the adjacent Z-line (Guo & Greaser, 2022). The myofibrillar regulatory protein composes of tropomyosin and troponin that regulate muscle contraction and relaxation. The myofibrillar structural proteins consist of Z-discs, M-line, costameres (ankyrin, spectrins, vinculin, talin, and gamma actin), intermediate filaments (desmin, filamin, spectrins, and ankyrin), titin, nebulin, and tropomodulin, which responsible to control the structure and integrity of myofibrils (Boland et al., 2019) (Figure 2.7).

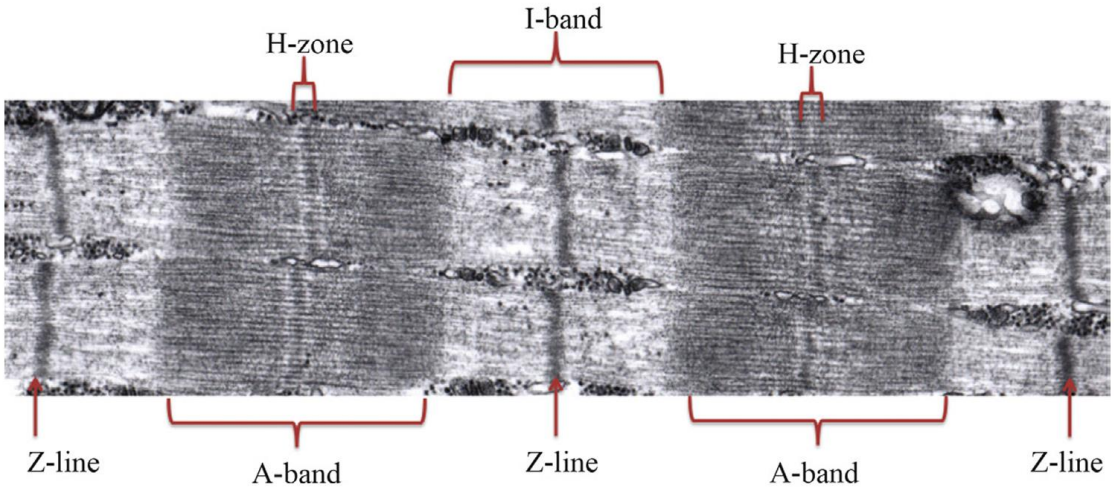

Figure 2. 6 An electron photomicrograph of a longitudinal section of a myofibril from skeletal muscle with specific areas and structures labelled (Lonergan, Topel, & Marple, 2019).

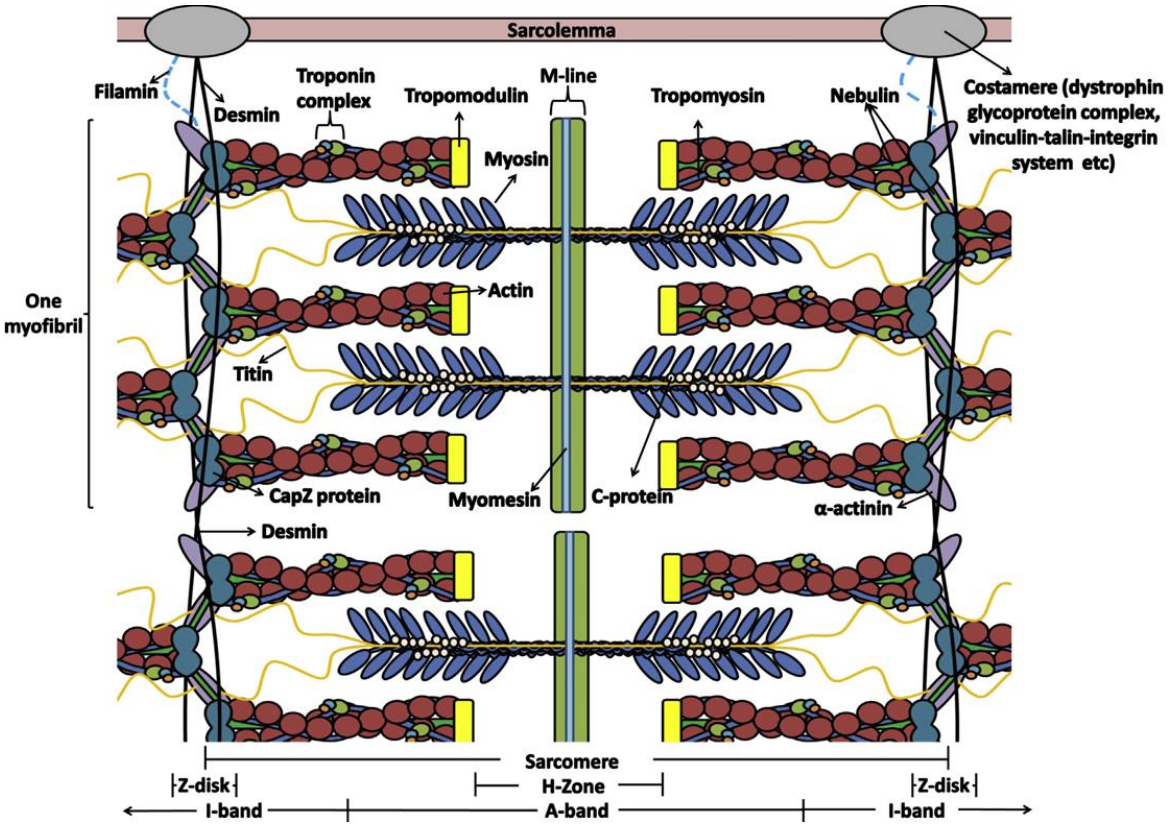

Figure 2. 7 Schematic model of the cytoskeletal protein structure (Boland et al., 2019).

2.3 Meat tenderness

Variation in tenderness among muscles can be expected since muscles themselves differ in their biological function (Calkins & Johnson, 2009). Figure 2.8 shows beef cuts and their tenderness classification. Muscles that are involved in locomotion are commonly found to be tougher compared to muscles that are used for support. Some tough cuts in the chuck and round area are ground and sold as minced meat for various purposes. Due to the versatility of ground meat, the meat attracts a higher price than some of the tender cuts per kg (Belew, Brooks, McKenna, & Savell, 2003).

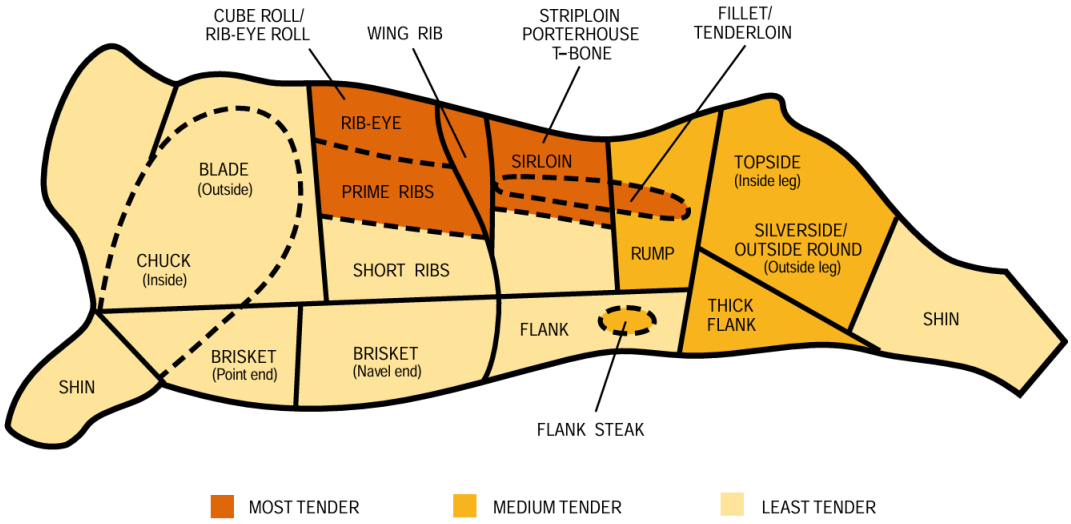

Figure 2. 8 Beef cut and tenderness ("Beef+Lamb New Zealand Reference Guide," 2010).

2.4 *Sous vide* and meat tenderness

Tenderness and juiciness are important meat quality attributes that determine consumer eating satisfaction (Becker et al., 2016). Furthermore, it has been shown that consumers are willing to pay more for tender meat (Miller, Carr, Ramsey, Crockett, & Hoover, 2001). Several studies have shown that *sous vide* cooking is able to improve the tenderness of tough meat cuts (Bhat, Morton, Zhang, Mason, & Bekhit, 2020; Botinestean, Keenan, Kerry, & Hamill, 2016; Christensen, Ertbjerg, Aaslyng, & Christensen, 2011; Fareh, 2018; Gámbaro et al., 2023; Naqvi et al., 2021).

Bhat et al. (2020) reported that beef semitendinosus *sous vide* cooked at 60 °C for 4.5 h and 10 h has significantly lower shear force value compared to that at 80 °C (the core temperature reached 75 °C). Similarly, Botinestean et al. (2016) demonstrated that *sous vide* cooking at 60 °C for 4.5 h reduced the shear force value of beef semitendinosus from 33 N (cooked at 75 °C) to 28 N. Additionally, (Christensen et al., 2011) reported that prolonged cooking time from 5 to 17 h at 53, 58, and 63 °C caused a reduction in shear force value of pork semitendinosus muscle from 39 N to 13 N. Using loin as the reference sample, Gámbaro et al. (2023) showed that *sous vide* cooking at 55 °C for 24 h resulted in beef shank with shear force value and sensory tenderness similar to loin. Furthermore, Naqvi et al. (2021) reported that prolonged cooking for 1, 8, and 18 h at 55,

65, and 75 °C resulted in a decreased shear force value of the beef biceps femoris and semitendinosus muscles. They also observed that muscle from older animals required higher temperatures and longer cooking times to achieve comparable tenderness to muscles from young animals.

The tenderness developed during *sous vide* cooking is primarily attributed to the solubilisation of collagen and the degradation of myofibrillar and connective tissue proteins by meat endogenous enzymes such as cathepsin B and L (Davey & Gilbert, 1976; Dominguez-Hernandez et al., 2018; Naqvi et al., 2021). Christensen et al. (2011) reported significant correlation between shear force value and the amount of solubilised collagen. Similarly, Wang et al. (2022) found that collagen solubility is one of the key factors that contribute to the tenderness of yak meat.

Cathepsin B and L has been associated to the development of tenderness during *sous vide* cooking mainly due to the thermal stability of these enzymes, which has been shown to remain active during *sous vide* cooking of meat. Besides, heat treatment also promotes the release of cathepsin B and L from lysosomes into the cytosol as has been demonstrated by the increase in their residual activity at the early heating treatment (Ertbjerg, Christiansen, Pedersen, & Christensen, 2012; Kaur et al., 2020).

2.5 *Sous vide* and cooking loss

Cooking loss is an important aspect of meat quality attributes that determine cooking yield and the sensory perception of cooked meat. During *sous vide* cooking of meat, the cook loss is retained within the vacuum bag. This retention of cook loss means that the product maintains its original weight after cooking, thus providing economic advantages to the industry. Despite this, the retention of high amount of cook loss inside the vacuum bag can impair the product's appearance which may discourage consumers from making a purchase (Szerman et al., 2008). Furthermore, high cooking loss is also correlated with reduced juiciness, leading to a decrease in consumer eating satisfaction (Aaslyng, Bejerholm, Ertbjerg, Bertram, & Andersen, 2003; Martens, Stabursvik, & Martens, 1982). Moreover, high cooking loss masks the benefit of meat

tenderisation, as meat with high cooking loss is rated to have low sensory tenderness and overall acceptability even though it has a low shear force value (Becker et al., 2016; Tkacz, Modzelewska-Kapituła, Petracci, & Zduńczyk, 2021). Thus, it is important to understand the factors that contribute to the variation in cook loss during *sous vide* cooking in order to improve the quality of *sous vide*-cooked meat.

Meat composes approximately 75% water (López-Bote, 2017). In pre-rigor meat, almost 85% of water is held by capillary force within the myofibrils, with a higher proportion is located within the less protein-dense I-band rather than the A-band (Bertram, Purslow, & Andersen, 2002; Huff-Lonergan & Lonergan, 2005). During rigor mortis, the reduction in the pH of meat to ~pH 5.5 (near the isoelectric point of myosin, pI 5.4) and the formation of actomyosin bridges lead to transverse and longitudinal shrinkage of the myofibrils, respectively. In the presence of intact cytoskeletal proteins (vinculin, desmin, and talin) that link myofibrils together and myofibrils to the sarcolemma (cell membrane), the transverse shrinkage of the myofibrils can be transmitted to the transverse shrinkage of the whole muscle cell. This, coupled with the increase in cell membrane permeability causes the expulsion of water along with water-soluble components from the myofibrillar space to the extramyofibrillar space (between the myofibrils and the sarcolemma) and extracellular space. The transverse shrinkage also leads the formation of drip channels, first observed between fibre bundles (perimysial network) and later between fibres (endomysial network). The formation of the drip channels allows the expelled fluid to flow out of the meat under gravitational force as drip loss, primarily occurs through channels between fibre bundles (Huff-Lonergan & Lonergan, 2005; Kristensen & Purslow, 2001; Offer & Cousins, 1992).

During cooking, heat induces the denaturation of meat proteins, leading to a further reduction in water-holding capacity and the contraction of meat protein networks. The transversal and longitudinal shrinkage of meat protein networks generate a pressure gradient within the meat and also widen the fluid channels already present during rigor mortis. This results in the expulsion of excess water, along with water-soluble components, to the surface of the meat as cook loss (Feyissa, Gernaey, & Adler-Nissen, 2013; Tornberg, 2005). The pronounced increase in meat

transverse shrinkage occurs at temperatures between 45 and 60 °C, attributed to myosin denaturation. Additionally, the longitudinal shrinkage begins at 60 – 65 °C and continues to increase until 90 °C, associated with the denaturation of actin and collagen (Vaskoska, Ha, Ong, Chen, et al., 2021; Vaskoska, Ha, Ong, Kearney, et al., 2021).

The degree of cooking loss depends on cooking temperature and time. The increase in cooking temperature from 45 to 80 °C causes a uniform increase in cooking loss until it levels off at temperatures > 80 °C (Martens et al., 1982; Vaskoska, Ha, Ong, Chen, et al., 2021). Prolonged cooking time also leads to an increase in cooking loss, primarily at temperatures < 70 °C (Zielbauer, Franz, Viezens, & Vilgis, 2016). Furthermore, meat from different muscle cuts shows varying amounts of cooking loss attributed to the differences in muscle type, pH, and collagen content. Table 2.3 summarises the effects of different factors on cooking loss in meat.

Table 2. 3 Factors affecting cooking loss in meat.

Factors	Sample and cooking treatment	Findings	Reference
Temperature	Beef *semimembranosus*, 1.5 years old bulls, *sous vide* cooked at 1.2 °C/ min to 45 – 85 °C, combine holding time of 5 and 20 min.	• Cooking loss increased uniformly (5 – 35 %) with an increase in temperature from 45 – 85 °C. • A marked decrease in sensory juiciness occurs between 63 and 73 °C, attributed to actin denaturation.	(Martens et al., 1982)
Temperature and time	Pork *psoas major*, *sous vide* cooked at 45 – 74 °C for 10 – 48 h.	• Cooking loss increased (8 – 30 %) with an increase in time and temperature, except at 74 °C, where cooking loss remained constant after 4 h. • A marked increase in cooking loss occurs between 60 and 74 °C, attributed to actin denaturation. • Meat proteins denatured completely at 74 °C in the shortest cooking time, but cooking loss still increased until 4 h, attributed to the slow dynamics of water transport.	(Zielbauer et al., 2016)
Muscle	Beef *masseter* (type I fibres, pH 6.3) and	• At temperatures < 80 °C, *masseter* had a lower cooking loss than	(Vaskoska, Ha, Ong,

	cutaneous trunci (type II fibres pH 5.6), *sous vide* cooked at 5°C/ min to the end-point temperatures of 50 – 85 °C.	*cutaneous trunci*, but at 85 °C both had similar cook loss (~22 %). • The lower cooking loss in masseter muscles was attributed to the greater water holding capacity of type I fibres and their high pH value.	Chen, et al., 2021)
Muscle and aging time	Beef *semitendinosus*, *biceps femoris* and *psoas major*, 1 and 14 days aging, 50, 60, 70 and 80 °C for 30 min.	• Aged muscle had a higher cooking loss compared to unaged muscle across all muscle and cooking temperature. • At 80 °C, *psoas major* had the lowest cooking loss compared to *biceps femoris* and *semitendinosus* muscle, attributed to the low collagen content in *psoas major*.	(Vaskoska, Ha, Naqvi, White, & Warner, 2020)
Animal age and aging time	Beef *semitendinosus* and *biceps femoris*, young (<1.5 years old) and old (2.5 – 3.5 years old), 0 and 13 days aging[a], *sous vide* cooked at 55, 65, and 75 °C for 1, 8, and 18 h.	• Cooking loss increased (13 – 41 %) with an increase in temperature and time, except at 75 °C, where cooking loss remained constant after 8 h. • Young animals had higher cooking loss compared to older animals. • Aging caused a decrease in total water content in meat from young animals, but not in older animals.	(Naqvi et al., 2021)
Species	Pork *semitendinosus*, beef *semitendinosus*, and chicken *pectoralis profundus*, *sous vide* cooked at 53 and 58 °C for 6, 17, and 30 h.	• Cooking loss increased with an increase in temperature and time, significant in beef and chicken. • Beef had the highest cooking loss (25 – 32 %), followed by pork (18 – 26 %), and was lowest in chicken (8 – 16 %).	(Christensen et al., 2012)
pH and water holding capacity of raw meat	Pork *longissimus dorsi*, pan and oven cooked (90 and 190 °C) to the end-point temperatures of 60, 70, and 80 °C.	• Meat with low water holding capacity and a low pH value (pH 5.3) had a higher cooking loss compared to meat with a high pH value (pH 6.0), but the difference became smaller with increasing cooking temperature.	(Aaslyng et al., 2003)

[a] fabrication was performed at 2-days postmortem.

2.6 Cathepsin B and L

Cathepsins (from the Greek word 'kathepsein,' meaning 'to digest') are a group of proteases with an acidic pH optimum that include serine proteases (cathepsin A and G), aspartic proteases (cathepsin E and D), and cysteine proteases (cathepsin B, C, F, H, K, L, O, S, V, X and W) (Turk et al., 2012). They are among the 50 known lysosomal hydrolases that are located inside lysosomes. Cathepsins are involved in both extracellular and intracellular protein degradation, which occurs through endocytosis and autophagy, respectively (Repnik, Hafner Česen, & Turk, 2014). The acidic environment of lysosomes (pH 4.0 – 5.5) allows for optimal cathepsin activity and promotes protein unfolding Additionally, lysosomes contain a high cysteine/cystine ratio, which is important to maintain the optimal activity of cysteine proteases and facilitate the breaking of protein disulfide bonds. These factors together provide efficient conditions for protein degradation inside the lysosome (Bechet, Tassa, Taillandier, Combaret, & Attaix, 2005; Pisoni, Acker, Lisowski, Lemons, & Thoene, 1990). In order to prevent uncontrolled protein degradation in the event of cathepsin escape from the lysosome, the cytosol contains endogenous cathepsin inhibitors, including cystatins, thyropins, and serpins. Among cathepsins, cathepsin B, H, L, C, X, F, O, V and D are present in all tissues, suggesting their important role in protein degradation. On the other hand, cathepsin K, W, and S have more specific tissue locations, suggesting their specific roles (Turk et al., 1999; Turk et al., 2012). In the area of meat science, cathepsin B, L, D, and H have been widely reported.

The stability of lysosomes is affected by several factors such as osmotic pressure, pH, temperature, salts, and freezing/ thawing (Gianetto & De Duve, 1955; Parrish & Bailey, 1967). The incubation of isolated lysosomes in 0.25 M sucrose produced a decrease in absorbance, measured by spectrophotometry, with increasing incubation time compared to lysosomes incubated in 0.7 M sucrose. This decrease is concomitant with an increase in lysosomal enzyme activity in the soluble fraction, suggesting that the reduction in osmotic pressure causes disruption of the lysosomal membrane, leading to the release of lysosomal enzymes (Sawant, Shibko, Kumta, & Tappel, 1964). Furthermore, increasing the incubation temperature of isolated

lysosomes from 4 to 45 °C, decreasing the pH from 7.0 to 3.0, and adding salts (NaCl and KCl) also result in an increase in lysosomal enzyme activity in the soluble fraction (Parrish & Bailey, 1967; Sawant, Desai, & Tappel, 1964; Welman & Peters, 1976).

In muscle fibres, lysosomes are primarily located at the periphery, with some also found in the myofibrillar space (O'Halloran, Troy, Buckley, & Reville, 1997; Okada, Robinson, & Karnovsky, 1986). Using cytochemical and immunohistochemical analyses, several studies have reported that aging, high-pressure treatment, electrical stimulation, and lactic acid injection can affect lysosomal membrane integrity. This is demonstrated by the discontinuities in lysosomal structure (white spots) and the diffusion of lysosomal enzymes in the vicinity of lysosomes and into the myofibrils of treated meat (Berge et al., 2001; Jung, de Lamballerie-Anton, Taylor, & Ghoul, 2000; Kubo et al., 2002; Li et al., 2012). This change in lysosomal membrane integrity was confirmed by biochemical analysis, as shown by the increase in cathepsin B, L, D, and acid phosphatase activity in the meat sarcoplasmic fraction after the treatment (Berge et al., 2001; Jung et al., 2000; Li et al., 2012). Additionally, the increase in lysosomal enzyme activity in meat sarcoplasmic extract was also observed in meat subjected to thermal treatment such as high-temperature aging (37 °C) and low-temperature cooking (Ertbjerg et al., 2012; Kaur et al., 2020; Wu, Dutson, & Carpenter, 1981). The release of cathepsins into the sarcoplasm allows them to come into contact with myofibrillar and connective tissue proteins, thereby having the potential to catalyse their degradation and contribute to meat tenderisation.

Cathepsins B, L, D, and H are endopeptidases, with cathepsins B and H also acting as exopeptidase and aminopeptidase, respectively. Cathepsins B and L have optimal activity at pH 5.5 – 6.5, while cathepsin D has a slightly lower optimal pH, which is at pH 3.0 – 5.0. In contrast, cathepsin H has an optimal pH of 6.5 – 6.8 (Salvesen & Rawlings, 2012; Sentandreu, Coulis, & Ouali, 2002). Cathepsin B, L, and D are thermally stable at temperatures below 60 °C, while cathepsin H is thermally labile (Kaur et al., 2020; Spanier, McMillin, & Miller, 1990) . Since cathepsins B and L have optimal activity at the pH found in post-mortem meat and are thermally

stable at temperatures below 60 °C, these enzymes are associated with meat tenderisation during low-temperature *sous-vide* cooking.

Cathepsin B and L can degrade myofibrillar proteins such as myosin heavy chain, actin, and troponin T. Additionally, cathepsin B can degrade desmin, while cathepsin L can degrade α-actinin and troponin I (Bandman & Zdanis, 1988; Baron, Jacobsen, & Purslow, 2004; Dufour, Ouali, Obled, Deval, & Valin, 1989; Huang, He, Li, & Yang, 2019; Matsukura, Okitani, Nishimuro, & Kato, 1981; Schwartz & Bird, 1977). Furthermore, cathepsin B can degrade insoluble collagen, resulting in an increased amount of solubilised collagen (Burleigh, Barrett, & Lazarus, 1974; Kirschke, Kembhavi, Bohley, & Barrett, 1982). Moreover, cathepsin L can cleave proteoglycans and glycosaminoglycans (GAG), leading to a decrease in the tensile strength of collagen fibres (Preety Panwar et al., 2013). Ultrastructural observation shows that the incubation of beef myofibrils with cathepsin B and L causes the disappearance of Z-line and M-line structures, as well as a decrease in the density of the A-band (Mikami, Whiting, Taylor, Maciewicz, & Etherington, 1987; Ouali et al., 1987). The degradation of these myofibrillar proteins has been linked to the weakening of the myofibrillar structure, which leads to improved meat tenderness. (Huff-Lonergan, Zhang, & Lonergan, 2010).

There is considerable evidence suggesting that cathepsin B and L contribute to meat tenderness. For example, a decrease in muscle fibres breaking stress was observed in isolated single muscle fibres incubated with cathepsin L at pH 5.6 at room temperature (Christensen, Larsen, Ertbjerg, & Purslow, 2004). Furthermore, greater tenderness and myofibrillar degradation were observed in meat with a low ultimate pH (≤ 5.79) and in meat injected with lactic acid, which correlates with a greater release of cathepsin B and L into the sarcoplasm (Berge et al., 2001; Lomiwes, Farouk, Frost, Dobbie, & Young, 2013). Similarly, the lower shear force value in meat subjected to electrical stimulation is associated with greater cathepsin B and L activity in the sarcoplasmic fraction (Li et al., 2012). Moreover, the greater tenderness in meat with a low ultimate pH compared to intermediate pH (5.8 – 6.3) can be explained by the increased degradation of M-lines and myosin heavy chain, which is linked to the action of cathepsins (Yu & Lee, 1986).

The contribution of cathepsin B and L to meat tenderness during *sous-vide* cooking has been demonstrated by a significant negative correlation between their activity and shear force values ($r = -0.50$; $p < 0.001$ and $r = -0.53$; $p \leq 0.05$) (Christensen et al., 2011; Dominguez-Hernandez & Ertbjerg, 2021). Furthermore, it has been shown that meat cooked at 53, 58, and 63 °C, has smaller particle sizes and lower shear force values compared to meat cooked at 68 and 73 °C. This is correlated with the presence of cathepsin B and L during the early heating time at low temperatures, which promotes greater myofibrillar proteolysis, thus preventing the formation of large aggregates as cooking time increases, resulting in tender meat (Dominguez-Hernandez & Ertbjerg, 2021). Moreover, greater tenderness was observed in meat precooked at 50 and 60 °C for 5 to 35 mins compared to meat cooked directly at 98 °C. This is associated with the presence of cathepsin B and L during the precooking stage, which contributes to the liberation of actin and consequently prevents shrinkage when the meat is further cooked at 98 °C, resulting in observed tenderness (Yao et al., 2023).

Thermal treatment causes the activation of cathepsin B and L, as demonstrated by an increase in their activity in the sarcoplasmic fraction. This increase in activity is associated with the effect of heat treatment on lysosomal membrane stability, which causes the release of cathepsin B and L into the sarcoplasm. Ertbjerg et al. (2012) reported a twofold increase in cathepsin B and L activity in pork *longissimus* muscle after cooking at 55 °C for 40 mins and 70 °C for 2 mins, compared to raw meat. Additionally, Kaur et al. (2020) reported a similar increase in cathepsin B and L activity in beef brisket after cooking at 50 °C for 1 h.

Since enzymes are proteins, thermal treatment also causes their deactivation. Hu, Zhou, Xu, Zhang, and Li (2023) demonstrated that heating cathepsin B at high temperatures resulted in a reduction of sulfhydryl groups and alterations in its secondary structure, leading to its deactivation. The decrease in cathepsin B and L activity in the sarcoplasmic fraction after heat treatment has been widely reported (Christensen et al., 2011; Dominguez-Hernandez & Ertbjerg, 2021; Ertbjerg et al., 2012; Kaur et al., 2020). For example, Kaur et al. (2020) reported a 50% reduction in cathepsin B activity in beef brisket after cooking at 50 °C for 24 h, relative to raw

meat. Furthermore, Dominguez-Hernandez and Ertbjerg (2021) reported a 40 % decrease in cathepsins B and L activity in pork *longissimus* muscle after cooking at 53 °C for 8 h, relative to 1 h.

However, no kinetic model has been developed to predict the thermal activation and deactivation of cathepsin B and L, especially in intact meat. Recently, a study was conducted to predict the thermal deactivation kinetics of cathepsin B in the sarcoplasmic extract of yellow-feathered chickens (Li et al., 2021). The results show an activation energy of (E_a) of 71.9 ± 3.2 kJ/mol and deactivation rate constants of 0.13, 0.24, and 0.29 min^{-1} at 50, 55, and 60 °C, respectively. Additionally, the study reported that at 50 °C, 21 mins are required to achieve 90 % deactivation of cathepsin B. This is markedly faster than what was reported by Kaur et al. (2020), who found that at 50 °C for 24 h, cathepsin B still retained 50 % of its activity. This suggests that the kinetic parameters of cathepsin B deactivation in sarcoplasmic extract may not accurately predict its activity in intact meat.

2.7 Two-stage *sous vide* cooking

Several studies have reported increased tenderness and juiciness with two-stage cooking/ *sous vide* cooking compared to conventional one-stage cooking/ *sous vide* cooking. Obuz et al. (2003) reported a lower shear force value in beef *biceps femoris* when cooked at 57 °C for 15 min and subsequently cooked at 71 °C, compared to meat directly cooked at 71 °C. Similarly, Powell, Dikeman, and Hunt (2000) demonstrated that beef *semitendinosus* muscle roasted in a conventional oven at 163 °C with a holding time at 55 – 65 °C for 1 h before cooking to the end temperature of 65 °C, has a lower shear force value than meat roasted directly to 65 °C.

Furthermore Ismail et al. (2019) reported a lower cooking loss in beef *semitendinosus* muscle *sous vide* cooked using two-stage *sous vide* cooking (49 °C for 3 h followed by 60 °C for 3 h, 45 °C for 6 h followed by 60 °C for 6 h, and 49 °C for 6 h followed by 65 °C for 6 h) compared to their corresponding single-stage *sous vide* cooking (60 °C for 6 h, 60 °C for 12 h, and 65 °C for 12 h) under similar total cooking time. However, the effect of two-stage *sous vide* cooking on

meat tenderness produced conflicting results. For example, two-stage cooking at 49 °C for 6 h followed by 60 °C for 6 h, and 49 °C for 6 h followed by 75 °C for 6 h, has a lower shear force value than their corresponding one-stage cooking at 60 °C for 12 h and 75 °C for 12 h. In contrast, two-stage cooking at 49 °C for 3 h followed by 60 °C for 3 h, and 49 °C for 6 h followed by 70 °C for 6 h, has a higher shear force value than their corresponding one-stage *sous vide* cooking at 60 °C for 6 h and 70 °C for 12 h.

Yao et al. (2023) reported a lower cooking loss and shear force value in pork *longissimus* muscle that was precooked at 50 °C for 35 min and at 60 °C for 5 and 20 min compared to those directly cooked at 100 °C. Furthermore, Uttaro, Zawadski, and McLeod (2019) reported that beef *supraspinatus* cooked using multistage *sous vide* cooking (M-SV; 1 h at 39 °C, 1 h at 49°C, 4 h at 59 °C) has a lower shear force value than that cooked using single-stage *sous vide* cooking (S-SV; 4 h at 59°C). They attributed the observed tenderness to the activity of calpains, the 20S proteasome, and cathepsins, which remain active during low-temperature cooking at 39 °C and 49 °C. However, they found that the results are muscle-dependent, with no effect observed for beef *rectus femoris*.

2.8 Gaps in the literature

Numerous studies have explored the use of two-stage cooking to tenderise meat and reported a greater tenderness and reduction in cooking loss with two-stage cooking than with single-temperature treatment (Ismail, Hwang, & Joo, 2019; Obuz, Dikeman, & Loughin, 2003; Walsh et al., 2010; Yao et al., 2023). However, optimal temperature-time combinations for maximizing the benefits of two-stage cooking have not been fully established. Additionally, the exact mechanism of tenderisation due to the two-stage cooking remains unclear, particularly in terms of how varying level of cathepsin B and L activity, collagen denaturation, and solubilisation during the first stage influence meat tenderness, cooking loss, and solubilised collagen of the final product, thus requiring further investigation. Furthermore, despite numerous studies has been done to determine cathepsin B and L activity during *sous vide* cooking of meat, no kinetic models have

been developed to predict their activity. Similarly, even though several studies has been done to characterise collagen thermal denaturation in cooked meat, no kinetic models have been developed, especially in intact meat (Christensen et al., 2013; Stabursvik & Martens, 1980; Zielbauer et al., 2016). The development of a kinetic model for cathepsin B and L activity and collagen denaturation can provide better insights into their involvement in the development of meat tenderness during *sous vide* cooking.

CHAPTER 3 Kinetic models to predict cathepsin B and L activity during *sous vide* cooking

3.1 Introduction

Sous vide is a cooking technique that involves heating vacuum-packed foods inside heat-stable plastic pouches under controlled temperature and time. The mechanisms that contribute to tenderisation during *sous vide* are still not yet fully understood. One of the mechanisms that has been suggested is due to the proteolysis of myofibrillar and connective tissue proteins by meat endogenous enzymes, such as cathepsin B and L (Dominguez-Hernandez et al., 2018). These enzymes have been reported to remain active during *sous vide* cooking of beef (Christensen et al., 2013; Kaur et al., 2020; Yin et al., 2020), pork (Christensen et al., 2011; Dominguez-Hernandez & Ertbjerg, 2021; Ertbjerg et al., 2012), and duck (Wang et al., 2013).

Cathepsin B and L are predominantly located inside lysosomes (cytoplasmic organelles) (Turk et al., 2012). Factors such as high temperature destabilise the lysosomal membrane, causing the release of lysosomal enzymes into the sarcoplasm (Sawant, Desai, et al., 1964; Zhang, Li, Vandepeer, & Zhao, 2006). The increase in the cathepsin B and L activity in the sarcoplasmic extract after thermal treatments has been reported by Ertbjerg et al. (2012) and Kaur et al. (2020). Once in the sarcoplasm and at the pH of normal post-mortem meat (pH 5.6), cathepsin B and L have the potential to promote myofibrillar and connective tissue proteins proteolysis.

Despite numerous studies has been done to characterise cathepsin B and L activity during *sous vide* cooking of meat, no kinetic models have been developed to predict their activity (Christensen et al., 2013; Dominguez-Hernandez & Ertbjerg, 2021; Ertbjerg et al., 2012; Kaur et al., 2020). Furthermore, the experimental data available in the literature is inadequate to be used for kinetic modelling especially the data on the activation phase (release of cathepsin B and L from lysosome). Recently, Li et al. (2021) reported the kinetics of cathepsin B thermal deactivation performed on sarcoplasmic extract obtained from yellow-feathered chickens. However, since the thermal stability of enzymes in enzyme extracts may be different from that in a food system, the

results obtained in the study may not accurately predict cathepsin B and L activity in meat during heat treatment (Van Boekel, 2009). Thus, the objective of this chapter is to develop a kinetic model to predict cathepsin B and L activity during *sous vide* cooking of beef brisket, which can be used as a first step to optimise the two-stage *sous vide* processing conditions.

3.2 Materials and methods

3.2.1 Chemicals

Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride (Z-Phe-Arg-AMC), Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-RR-AMC), 7-amino-4-methylcoumarin (AMC), and Pefabloc® SC were purchased from Sigma–Aldrich (New Zealand Co.). All chemicals were of analytical grade.

3.2.2 Sample preparation

Two beef briskets (5 days post-mortem) were purchased from a local butcher in two separate weeks, one in March 2021 (Brisket 1) and another one in April 2021 (Brisket 2). The external fat and connective tissue were first trimmed off from each brisket that was then cut across the muscle fibre into slices (Figure 3.1) of 2 mm in thickness. A thin size of the meat samples was selected to allow rapid heating and a near isothermal condition during *sous vide* cooking. The slices were then individually vacuum packed and stored overnight at 4 °C before being processed on the next day.

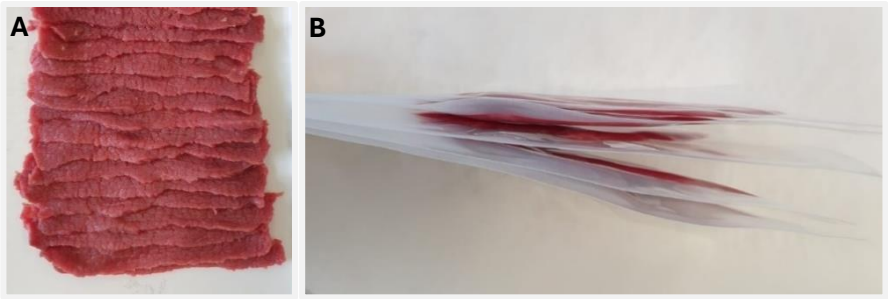

Figure 3. 1 Sample of beef brisket with 2mm thickness. A) Sample before vacuum-packaging; B) Sample after vacuum-packaging.

Cathepsin thermal kinetic study was performed by cooking the meat samples at temperatures in the range of 46 – 60 °C for times ranging from 30 s – 72 h in temperature-controlled water baths. Specifically, the samples from Brisket 1 were *sous vide* treated at the temperatures of 46 °C, 48

°C, and 50 °C for times ranging from 30 sec – 18 h, making a total of 46 temperature-time combinations. The samples from Brisket 2 were *sous vide* treated at 48 °C, 50 °C, 52 °C, 54 °C, 56 °C, 58 °C and 60 °C for times in the range of 30 sec – 72 h, making a total of 55 temperature-time combinations. This gave total 101 temperature-time combinations. Cooking was stopped by immersing samples into ice water baths.

3.2.3 pH measurement

The pH value of the meat samples was measured using a pH meter (Orion 3 Star pH Benchtop, Thermo Electron Corporation) equipped with a glass electrode and a temperature sensor. Prior to measurement, the pH meter was calibrated with pH 4 and pH 7 standard solutions. The meat pH measurement was done by inserting the glass electrode and temperature sensor into the centre of the sample. Three replicate measurements were made for each brisket (Korkeala, Mäki-Petäys, Alanko, & Sorvettula, 1986).

3.2.4 Enzyme extraction

Enzyme extraction was performed according to the method of (Koohmaraie & Kretchmar, 1990). First, raw and cooked meat samples were finely chopped using a sharp knife. For the cooked samples, the finely chopped meat residue fraction was then recombined with the cook loss and mixed well. After that, 3 g of each sample were homogenized in 9 ml of the ice-cold buffer (50 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 0.15 M KCl and 1 mM EDTA) using Heidolph DIAX 900 (4 x 5 s, medium speed). The mixture was then centrifuged at 25,000 x g for 20 min at 4 °C (Thermo Scientific™ Sorvall™ RC 6 Plus). The supernatant was filtered using a 0.45 µm syringe filter and aliquoted into 1.5 ml Eppendorf tubes and immediately stored at -20 °C until further analysis. The supernatant (also known as sarcoplasmic fraction or enzyme extract) was used to: 1) measure the residual active cathepsin B and L of the meat samples and, 2) study the effect of temperature on their rate of reaction.

3.2.5 Determination of the effect of *sous vide* cooking on cathepsin B and L activity

Z-Phe-Arg-AMC is a common substrate for Cathepsin B and L (Barrett & Kirschke, 1981). These two enzymes catalyse hydrolysis of Z-Phe-Arg-AMC, liberating AMC that can be monitored by fluorescence at the wavelength at 460 nm upon excitation at 360 nm wavelength (Barrett, 1980).

In this experiment, enzyme extract obtained from raw and cooked beef brisket was measured under standardized experimental conditions (temperature 37°C, buffer, and substrate concentration). For this reason, it was assumed that the measured cathepsin B and L activity was directly proportional to the concentration of active cathepsin B and L in the sample.

Determination of Cathepsin B and L activity was performed by continuous assays as previously described (Kirschke, Wood, Roisen, & Bird, 1983) with some modifications. The assays were done on 96-well microplate (Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated). Each reaction contains 25 µl of the enzyme extract, 125 µl of the assay buffer (250 mM sodium acetate buffer, pH 5.5, containing 2.5 mM Na_2EDTA, 5 mM DTT, and 1 mM Pefabloc ®SC) and 100 µl of the enzyme substrate (Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride, 20 µM). The enzyme substrate was added last to initiate the reaction. The reaction mixture was shaken well for 5s and incubated at 37 °C in a multimode microplate reader (Varioskan Lux, Thermo Fisher Scientific Ltd., USA). Fluorescence was measured for every 1 min for a 10-min period (excitation wavelength: 360 nm, emission wavelength: 460 nm). The assays were done in duplicate. A negative control a blank sample, was included by substituting the enzyme extract with the homogenization buffer.

The same method was used for determination of cathepsin B activity except substrate Z-RR-AMC and buffer (250 mM sodium phosphate buffer, pH 6.0, containing 2.5 mM Na_2EDTA, 5 mM DTT, and 1 mM Pefabloc ®SC) were used for the enzyme assay.

3.2.6 Determination of the effect of temperature on cathepsin B and L rate of reaction

In this experiment, only one enzyme extract was selected for the enzymatic assays, so that the enzyme concentration is fixed across the temperature conditions. The effect of temperature on

cathepsin B and L reaction rate was performed using stopped assays as previously described (Kirschke et al., 1983) with slight modifications. The enzyme extract, 25 µl, was first added into 125 µl of the assay buffer (250 mM sodium acetate buffer, pH 5.5, containing 2.5 mM Na_2EDTA, 5 mM DTT, and 1 mM Pefabloc ®SC) in 1.5 ml microcentrifuge tube. This microcentrifuge tube containing the reaction mixture and the other tube containing 100 µl of the enzyme substrate (Z-Phe-Arg-AMC, 20 µM) was then incubated at a specific temperature (37, 40, 45, 50, 55, 60 °C) for 1 min in a temperature-controlled water bath. The pre-incubated substrate (100 µl) and the enzyme extract/buffer was mixed, gently vortexed, and immediately incubated at the corresponding temperature (37, 40, 45, 50, 55, 60 °C) at which they had been pre-incubated. The reaction was stopped by adding 250 µl of the stop solution (400 mM monochloroacetic acid in 400 mM sodium acetate buffer, pH 4.3) after incubation for 0, 1, 2, 3, 4, 5, 6, and 7 min. The mixture was gently vortexed, of which 250 µl was then transferred into a 96-microwell plate for fluorescence measurement at excitation and emission wavelength of 360 nm and 460 nm, respectively (Varioskan Lux Multimode Microplate Reader 3020-1333, Thermo Fisher Scientific Ltd., USA). A negative control, used as a blank sample, was included by substituting the enzyme extract with the homogenization buffer. Each treatment was done in triplicate.

3.2.7 Standard curve

A standard curve was constructed by measuring the fluorescence of known concentrations of the cathepsin-catalysed substrate hydrolysis product, AMC. Standard solutions at the concentrations of 2.5, 5, 7.5, 10, 12.5 µM were prepared from the AMC stock solution (50 mM in DMSO) using the 0.01 % CHAPS solution as the diluent. Standard curves for continuous and stopped assays were performed following the methods described in 3.2.5 and 3.2.6, respectively, in which the homogenization buffer and AMC solution at a pre-defined concentration was used in place of the enzyme extract and the substrate. A blank was created by substituting AMC with 0.01 % CHAPs. Overall, the final concentration of AMC per well was 0.25, 0.50, 0.75, 1.0, and 1.25 nmoles for the continuous assays and 0.125, 0.250, 0.375, 0.500, and 0.625 nmoles for the stopped assays. Each treatment was done in triplicate. After blank subtraction, the standard curve was constructed

by plotting the fluorescence *vs.* AMC concentration. The slope of standard curves was calculated using linear regression (Microsoft® Excel®). Figure 3.2 shows the standard curves for continuous and stopped assay in this study.

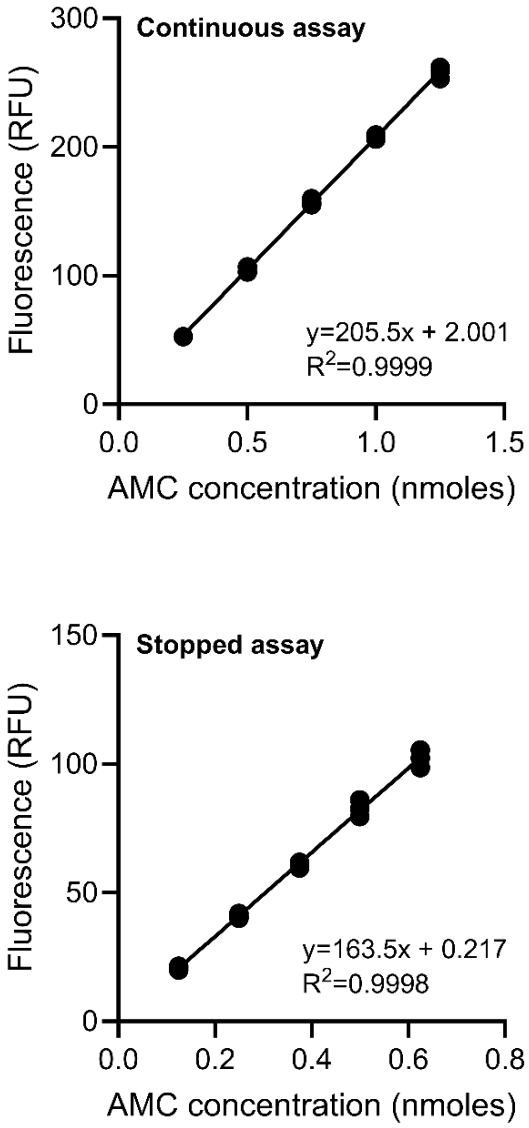

Figure 3. 2 Standard curves of cathepsin-catalysed reaction product, AMC, for continuous and stopped assays.

3.2.8 Calculation of cathepsin B and L activity

The fluorescence data in RFU (relative fluorescence units) obtained in the experiment was converted to the concentration of AMC using the standard curve. Cathepsin B and L activity was

expressed as U (Unit) where 1 U is defined as the amount of cathepsin B and L required to release 1 nmol of AMC per min. The amount of AMC per min was calculated from the initial linear part of the reaction progress curve using linear regression (Microsoft® Excel®) since during the enzyme reaction when the curve is linear the rate is dependent on the concentration and activity of the enzyme, the nonlinear section typically indicates the reaction is limited by the substrate concentration. Cathepsin B and L activity was also expressed as U per gram of meat.

$$\boldsymbol{Cathepsin\ activity\ per\ gram\ of\ meat\ (Ug^{-1}meat)} =$$

$$\frac{\boldsymbol{Cathepsin\ activity\ (U) x\ DF(ml.g^{-1})}}{\boldsymbol{V(ml)}} \quad \textbf{(3.1)}$$

Where:

DF = Dilution factor (total volume of homogenate in ml per sample weight in g)

V = Volume of enzyme extract used in the assay

3.2.9 Kinetic and data analysis

The kinetics of cathepsin B and L activity during *sous vide* cooking was modelled assuming consecutive reactions in series which are activation followed by deactivation (Figure 3.3). Activation can be defined as the release of cathepsin from lysosome to the sarcoplasm due to heat treatment. The released cathepsin is able to be in contact with meat myofibrillar and connective tissue protein thus has potential to catalyse their degradation. On the other hand, deactivation was defined as the denaturation of cathepsin due to the heat treatment. Cathepsin inside the lysosome, released cathepsin, and denatured cathepsin were designated as inactive cathepsin ([I]), active cathepsin ([A]), and denatured cathepsin ([D]), respectively. Two kinetic models were used to describe these consecutive reactions.

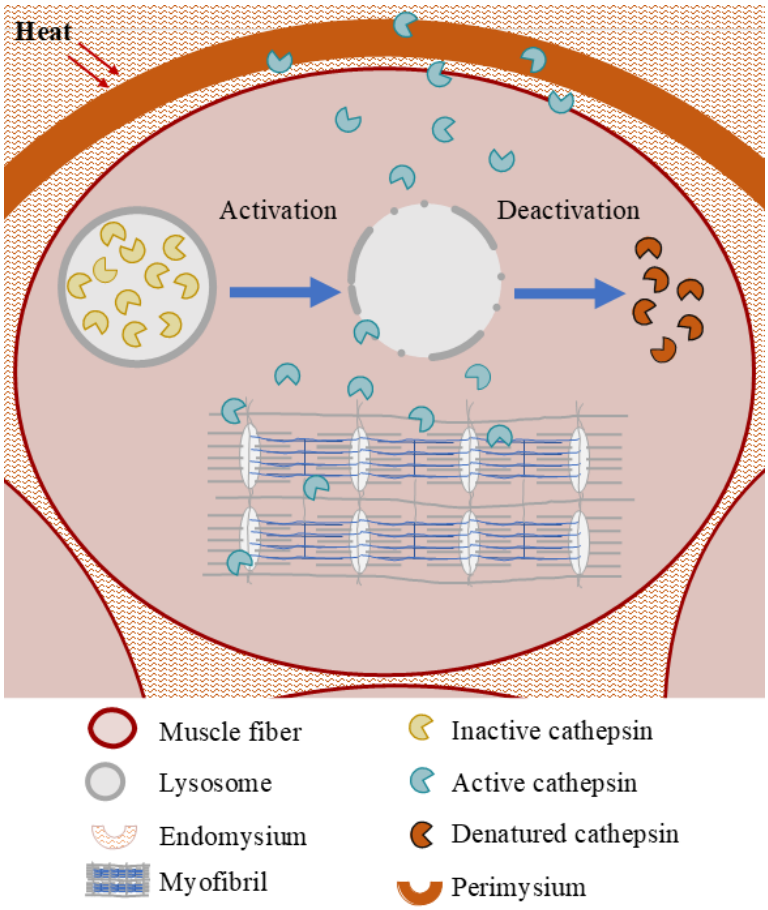

Figure 3. 3 Proposed model of cathepsin activation and deactivation during *sous vide* cooking.

3.2.9.1 Model 1

The kinetic of activation and deactivation of cathepsin were described using first-order reactions.

$$[\boldsymbol{I}] \overset{\boldsymbol{k_a}}{\rightarrow} [\boldsymbol{A}] \overset{\boldsymbol{k_d}}{\rightarrow} [\boldsymbol{D}] \quad \textbf{(3.2)}$$

Where, [I] = inactive cathepsin, [A] = active cathepsin, [D] = denatured cathepsin, k_a = rate of activation, k_d = rate of deactivation.

$$\frac{\boldsymbol{d[I]}}{\boldsymbol{dt}} = -\boldsymbol{k_a}[\boldsymbol{I}] \quad \textbf{(3.3)}$$

$$\frac{\boldsymbol{d[A]}}{\boldsymbol{dt}} = \boldsymbol{k_a}[\boldsymbol{I}] - \boldsymbol{k_d}[\boldsymbol{A}] \quad \textbf{(3.4)}$$

Integrating [I] with respect to t.

$$\int \frac{\boldsymbol{1}}{\boldsymbol{[I]}} \boldsymbol{d[I]} = -\boldsymbol{k_a} \int \boldsymbol{dt}$$

$$\ln[I] = -k_a t + C$$

C is integration constant, At t=0, $[I]=[I]_0$, therefore,

$$C = \ln[I]_0$$

$$\ln[I] - \ln[I]_0 = -k_a t$$

$$\ln \frac{[I]}{[I]_0} = -k_a t$$

$$\frac{[I]}{[I]_0} = e^{-k_a t}$$

$$[I] = [I]_0 e^{-k_a t} \quad \textbf{(3.5)}$$

Plugging Equation (3.3) into (3.4):

$$\frac{d[A]}{dt} = k_a\left([I]_0 e^{-k_a t}\right) - k_d[A]$$

$$\frac{d[A]}{dt} + k_d[A] = k_a\left([I]_0 e^{-k_a t}\right)$$

Multiply both side with integration factor.

$$\left(\frac{d[A]}{dt} + k_d[A]\right) x\, e^{k_d t} = \left(k_a[I]_0 e^{-k_a t}\right) x\, e^{k_d t}$$

$$e^{k_d t}\frac{d[A]}{dt} + k_d[A]e^{k_d t} = k_a[I]_0 e^{(k_d - k_a)t}$$

Since,

$$v\frac{du}{dx} + u\frac{dv}{dx} = \frac{d(v.u)}{dx}$$

Thus,

$$\frac{d\left([A]e^{k_d t}\right)}{dt} = k_a[I]_0 e^{(k_d - k_a)t}$$

Integrating $[A]e^{kdt}$ with respect to t.

$$\int d[A]e^{k_d t} = k_a[I]_0 \int e^{(k_d - k_a)t}$$

$$[A]e^{k_d t} = k_a[I]_0 \frac{e^{(k_d - k_a)t}}{(k_d - k_a)} + C$$

C is integration constant, At t=0, $[A]=[A]_0$, therefore,

$$[\boldsymbol{A}]_0\boldsymbol{e}^{\boldsymbol{0}} = \boldsymbol{k_a}[\boldsymbol{I}]_0\frac{\boldsymbol{e}^{\boldsymbol{0}}}{(\boldsymbol{k_d}-\boldsymbol{k_a})} + \boldsymbol{C}$$

$$\boldsymbol{C} = \frac{-\boldsymbol{k_a}[\boldsymbol{I}]_0}{(\boldsymbol{k_d}-\boldsymbol{k_a})} + [\boldsymbol{A}]_0$$

$$[\boldsymbol{A}]\boldsymbol{e}^{\boldsymbol{k_d t}} = \boldsymbol{k_a}[\boldsymbol{I}]_0\frac{\boldsymbol{e}^{(\boldsymbol{k_d}-\boldsymbol{k_a})\boldsymbol{t}}}{(\boldsymbol{k_d}-\boldsymbol{k_a})} - \frac{\boldsymbol{k_a}[\boldsymbol{I}]_0}{(\boldsymbol{k_d}-\boldsymbol{k_a})} + [\boldsymbol{A}]_0$$

$$[\boldsymbol{A}]\boldsymbol{e}^{\boldsymbol{k_d t}} = \frac{\boldsymbol{k_a}[\boldsymbol{I}]_0}{(\boldsymbol{k_d}-\boldsymbol{k_a})}\left[\boldsymbol{e}^{(\boldsymbol{k_d}-\boldsymbol{k_a})\boldsymbol{t}} - \boldsymbol{1}\right] + [\boldsymbol{A}]_0$$

$$[\boldsymbol{A}] = \frac{\boldsymbol{k_a}[\boldsymbol{I}]_0}{(\boldsymbol{k_d}-\boldsymbol{k_a})}\left[\boldsymbol{e}^{\boldsymbol{k_d t}-\boldsymbol{k_a t}}.\boldsymbol{e}^{-\boldsymbol{k_d t}} - \boldsymbol{e}^{-\boldsymbol{k_d t}}\right] + [\boldsymbol{A}]_0\boldsymbol{e}^{-\boldsymbol{k_d t}}$$

$$[\boldsymbol{A}] = \frac{\boldsymbol{k_a}[\boldsymbol{I}]_0}{(\boldsymbol{k_d}-\boldsymbol{k_a})}\left[\boldsymbol{e}^{-\boldsymbol{k_a t}} - \boldsymbol{e}^{-\boldsymbol{k_d t}}\right] + [\boldsymbol{A}]_0\boldsymbol{e}^{-\boldsymbol{k_d t}} \qquad \textbf{(3.6)}$$

Time when the A is maximum ($[A]_{max}$) was described using equation (3.7). At $t=t_{max}$, $A=[A]_{max}$:

$$\frac{\boldsymbol{d}[\boldsymbol{A}]}{\boldsymbol{dt}} = \boldsymbol{0}$$

$$\frac{\boldsymbol{k_a}[\boldsymbol{I}]_0}{(\boldsymbol{k_d}-\boldsymbol{k_a})}\left[\boldsymbol{e}^{-\boldsymbol{k_a t_{max}}} - \boldsymbol{e}^{-\boldsymbol{k_d t_{max}}}\right] + [\boldsymbol{A}]_0\boldsymbol{e}^{-\boldsymbol{k_d t_{max}}} = \boldsymbol{0}$$

$$\frac{\boldsymbol{k_a}[\boldsymbol{I}]_0}{(\boldsymbol{k_d}-\boldsymbol{k_a})}\left[\boldsymbol{e}^{-\boldsymbol{k_a t_{max}}} - \boldsymbol{e}^{-\boldsymbol{k_d t_{max}}}\right] = \boldsymbol{0}$$

$$-\boldsymbol{k_a}\boldsymbol{e}^{-\boldsymbol{k_a t_{max}}} + \boldsymbol{k_d}\boldsymbol{e}^{-\boldsymbol{k_d t_{max}}} = \boldsymbol{0}$$

$$\boldsymbol{k_a}\boldsymbol{e}^{-\boldsymbol{k_a t_{max}}} = \boldsymbol{k_d}\boldsymbol{e}^{-\boldsymbol{k_d t_{max}}}$$

$$\frac{\boldsymbol{e}^{-\boldsymbol{k_a t_{max}}}}{\boldsymbol{e}^{-\boldsymbol{k_d t_{max}}}} = \frac{\boldsymbol{k_d}}{\boldsymbol{k_a}}$$

$$\boldsymbol{e}^{\boldsymbol{t_{max}}(\boldsymbol{k_d}-\boldsymbol{k_a})} = \frac{\boldsymbol{k_d}}{\boldsymbol{k_a}}$$

$$(\boldsymbol{k_d}-\boldsymbol{k_a})\boldsymbol{t_{max}} = \boldsymbol{ln}\frac{\boldsymbol{k_d}}{\boldsymbol{k_a}}$$

$$\boldsymbol{t_{max}} = \frac{\boldsymbol{1}}{(\boldsymbol{k_d}-\boldsymbol{k_a})}\boldsymbol{ln}\frac{\boldsymbol{k_d}}{\boldsymbol{k_a}} \qquad \textbf{(3.7)}$$

3.2.9.2 Model 2

The kinetics of activation of cathepsin B and L was described using a first-order reaction while the deactivation was described following the deactivation kinetics of two groups of isozymes (Aa and Ab, where Aa + Ab=1) with different thermal stability, each deactivated according to a first-order reaction.

$$[\boldsymbol{I}] \overset{\boldsymbol{k_a}}{\rightarrow} [\boldsymbol{A}] \begin{cases} [\boldsymbol{A_a}] \overset{\boldsymbol{k_{dAa}}}{\longrightarrow} [\boldsymbol{D_a}] \\ [\boldsymbol{A_b}] \underset{\boldsymbol{k_{dAb}}}{\longrightarrow} [\boldsymbol{D_b}] \end{cases} \quad \textbf{(3.8)}$$

Where, [I] = inactive cathepsin, k_a = rate of activation, [A] = active cathepsin, [A_a] = the proportion of the active cathepsin isoenzyme A_a, [A_b] = the proportion of the active cathepsin isoenzyme A_b, (1 – A_a), k_{dAa} = rate of deactivation of isoenzyme A_a, k_{dAb} = rate of deactivation of isoenzyme A_b, [D_a] = the proportion of the inactivate cathepsin isoenzyme A_a, [D_b] = the proportion of the inactivate cathepsin isoenzyme A_b

For system that consist of classical single thermal stability fraction, the deactivation can be described as:

$$[\boldsymbol{A}] \overset{\boldsymbol{k_d}}{\rightarrow} [\boldsymbol{D}]$$

For first order reaction, at t=0, [A]=$[A]_0$, therefore:

$$\frac{[\boldsymbol{A}]}{[\boldsymbol{A}]_0} = \boldsymbol{e^{-k_d t}} \quad \textbf{(3.9)}$$

In the case of two distinct thermal stability fraction, the deactivation can be described as Aymard and Belarbi (2000):

$$[\boldsymbol{A_a}] \overset{(\boldsymbol{k_{dAa}})}{\longrightarrow} [\boldsymbol{D_a}]$$

$$[\boldsymbol{A_b}] \overset{(\boldsymbol{k_{dAb}})}{\longrightarrow} [\boldsymbol{D_b}]$$

For first order reaction, at t=0, [A]=$[A]_0$, therefore:

$$\frac{[\boldsymbol{A}]}{[\boldsymbol{A}]_0} = \boldsymbol{A_a e^{-k_{dAa} t} + (1 - A_a) e^{-k_{dAb} t}} \quad \textbf{(3.10)}$$

Comparing Equation (3.10) with (3.9) gave;

$$e^{-k_d t} = A_a e^{-k_{dAa} t} + (1 - A_a) e^{-k_{dAb} t} \quad \textbf{(3.11)}$$

and,

$$k_d = A_a k_{dAa} + (1 - A_a) k_{dAb} \quad \textbf{(3.12)}$$

To justify this, for system that does not consist of two distinct thermal stability fractions, A_a will equal to 1 and parameter A_b which is $(1 - A_a)$ will drop out. As a consequence, e^{-kdt} and k_d will return to single thermal stability fraction.

Thus, Model 2 was developed by substituting e^{-kdt} and k_d in Equation (3.6) following Equation (3.11) and (3.12), respectively.

$$[A] = \frac{k_a [I]_0}{(A_a k_{2Aa} + (1 - A_a) k_{2Ab}) - k_a} \left[e^{-k_a t} - \left(A_a e^{-k_{dAa} t} + (1 - A_a) e^{-k_{dAb} t} \right) \right]$$

$$+ [A]_0 \left(A_a e^{-k_{dAa} t} + (1 - A_a) e^{-k_{dAb} t} \right) \quad \textbf{(3.13)}$$

3.2.10 Data fitting and goodness of fit

The effect of temperature on reaction rate constants can be described using the Arrhenius equation (3.14). In this study, experimental data from different temperature-time treatments were fit simultaneously to Equation (3.6) (Model 1) and Equation (3.13) (Model 2) using reparameterization of the Arrhenius equation (3.15). The temperature of 52°C was chosen as reference (T_{ref}) since it was close to the middle of temperature range used in this study. k_{ref} is the reaction rate constant at T_{ref}. E_a is the activation energy of the reaction, R (8.31432 $J \cdot K^{-1} \cdot mol^{-1}$) is the universal gas constant, T is absolute temperature (K), and A is a pre-exponential factor.

$$.k = A e^{\frac{-E_a}{RT}} \quad \textbf{(3.14)}$$

$$k = k_{ref} e^{\left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right]} \quad \textbf{(3.15)}$$

The nonlinear least-squares fitting was carried out using Solver (Microsoft® Excel®) by changing the value of fitting parameters to minimise the sum of squared residual (value of 0 was chosen in

the *equal to* selection) between predicted and experimental data. Model 1 contains five fitting parameters and Model 2 contains eight fitting parameters. The parameter $[A]_0$ in each model was fixed to the value of the concentration of active cathepsins B and L in raw beef brisket.

The goodness of fit of the model was determined using the coefficient of determination (R^2), where $\bar{A}$ is the average of value of measured active cathepsin and $\hat{A}_{predicted}$ is the prediction of $A_{observed}$ (measured active cathepsin).

$$\boldsymbol{R^2 = 1 - \frac{\sum(A_{observed} - \hat{A}_{predicted})^2}{\sum(A_{observed} - \bar{A})^2}} \quad \textbf{(3.16)}$$

3.2.11 Determination of potential cathepsin B and L activity during *sous vide* cooking

The effect of temperature on the reaction rate (k) of the cathepsins B and L was described by plotting k against the temperature from the stopped assay data. An Arrhenius plot of natural logarithm of k (ln k) against the reciprocal of the temperature (Kelvin) (1/T) from temperature 37 and 55 °C was then constructed. The slope of the Arrhenius plot was obtained using linear regression (Microsoft® Excel®) and used to calculate the activation energy (E_a).

The obtained activation energy (E_a) was used to calculate A* (cathepsin B and L activity obtained by combining the effect of temperature on the cathepsin B and L activity and rate of reaction) during *sous vide* cooking (Equation 3.17).

$$\boldsymbol{A^* = Ae^{\left[\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{37°C}}\right)\right]}} \quad \textbf{(3.17)}$$

Where, A = cathepsin B and L activity, E_a = the activation energy of the reaction, and R = universal gas constant (8.31432 $J \cdot K^{-1} \cdot mol^{-1}$).

Cumulative cathepsin B and L activity was calculated from the area under the curve of the A* versus time using cumtrapz function (Matlab R2020b). Maximum cumulative activity and its corresponding temperature optimum at a specific time range was obtained using max function (Matlab R2020b). The graphs of cumulative cathepsin B and L activity and temperature optimum were plotted using Matlab R2020b.

3.2.12 Statistical analysis

All figures in this study were created using GraphPad Prism v9.4.0 unless mentioned otherwise. Two-sample t-test by Minitab® v18.1 was used to determine the statistical significance of difference between samples (Brisket 1 and 2).

3.3 Results and discussion

3.3.1 Kinetics of activation and deactivation of cathepsin B and L

Table 3.1 presents the characteristics of the beef brisket used in this study. Both Brisket 1 and Brisket 2 have pH values within the range of normal post-rigor pH values of beef. There were no significant differences in cathepsins B and L activity between the two brisket samples ($p = 0.235$) (Table 3.1). As a result, the kinetic data obtained from these two samples can be used for model fitting without normalizing it to their activities in the raw sample. It is worth noting that the cathepsin B and L activity in raw meat obtained in the present study falls within the range reported by other studies (Ertbjerg et al., 2012; Ertbjerg, Larsen, & Moøller, 1999).

Table 3. 1 The pH and cathepsin B and L activity of raw beef brisket.

Sample	pH[a]	Cathepsin B and L activity (U g^{-1}meat)[b]
Brisket 1	5.57 ± 0.02	1.34 ± 0.08
Brisket 2	5.55 ± 0.04	1.17 ± 0.21
Average	5.56	1.25

Each value represents mean ± standard deviation, (n = 3)[a] and (n = 4)[b].
1 U = 1 nmole AMC released per min at 37 °C.

Figure 3.4 illustrates the effect of temperature and time on cathepsin B and L activity. It is evident that cathepsin B and L remain active within the temperature range used in this study. The figure demonstrates that, across all temperatures, the activity of cathepsin B and L increase rapidly, reaching a peak nearly four times higher than their initial activity (raw beef brisket) at the beginning of the heat treatments, before decreasing at longer heating times. This observation supports the consecutive reactions model, which involves activation followed by deactivation as proposed in this study. A similar pattern was noted by Ertbjerg et al. (2012) and Kaur et al. (2020). The increase in cathepsin B and L activity indicates the release of these enzymes from lysosomes to the sarcoplasmic fraction due to the heat treatment. However, other factors such as heat-induced activation of cathepsin pro-enzymes have also been suggested (Ertbjerg et al., 2012).

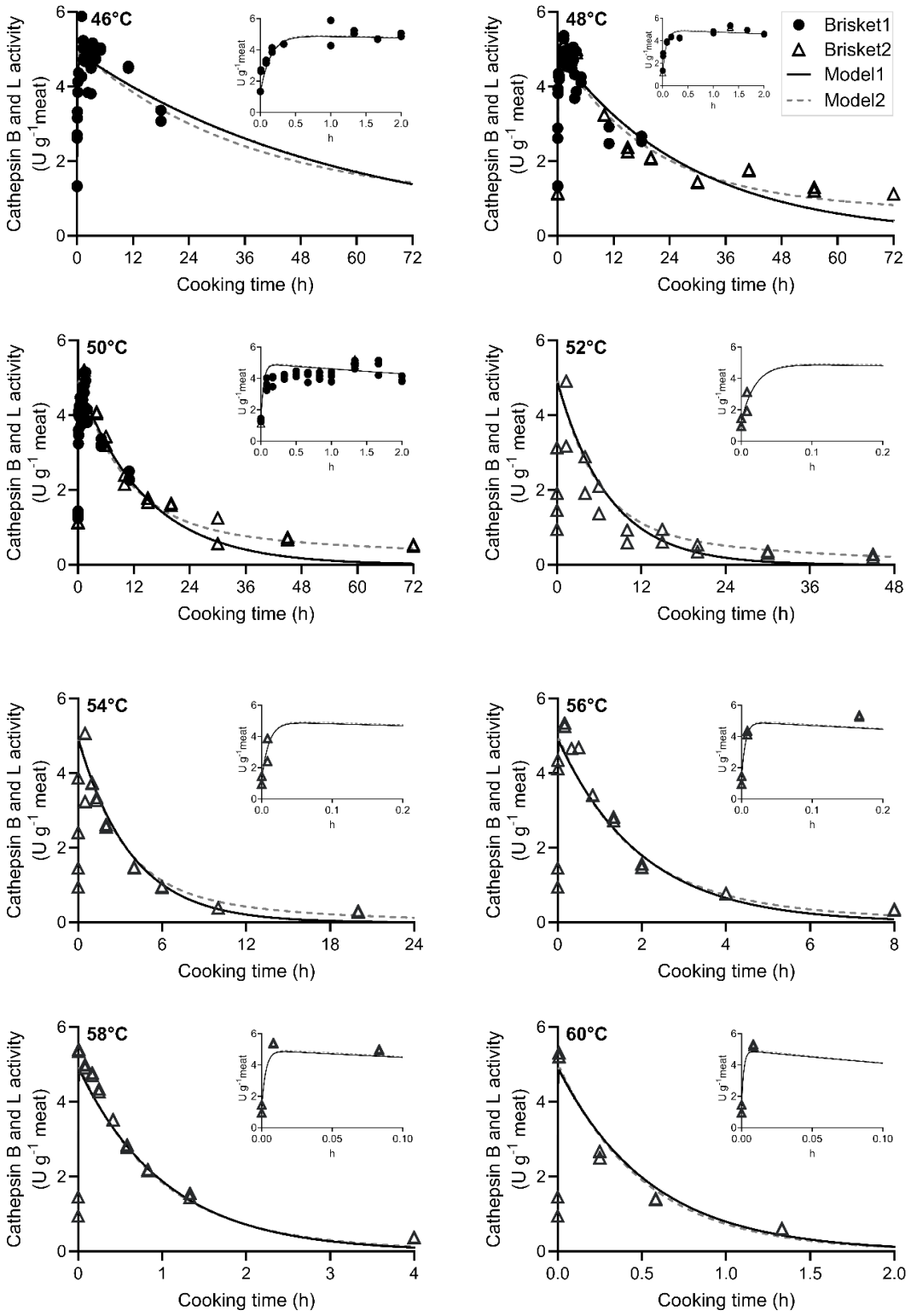

Figure 3. 4 Predicted (Model 1 and Model 2) and measured cathepsin B and L activity in beef brisket *sous vide* cooked at 46 – 60 °C for 0 – 72 h. For each temperature, the inset depicts the activation phase. 1 U is equal to 1 nmoles AMC released per min at 37 °C. Brisket 1 and Brisket 2 refer to meat that was purchased in two separate weeks.

Table 3. 2 Estimated fitting parameters obtained from Model 1 and Model 2.

Parameter	Value	Unit
Model 1		
$[I]_0$	3.67	U g^{-1}meat
E_a	287	kJ mol^{-1}
$k_{a\_52°C}$	0.8403	min^{-1}
E_d	293	kJ mol^{-1}
$k_{d\_52°C}$	0.0023	min^{-1}
R^2	0.900	
Model 2		
$[I]_0$	3.76	U g^{-1}meat
E_a	284	kJ mol^{-1}
$k_{a\_52°C}$	0.8040	min^{-1}
E_{dAa}	270	kJ mol^{-1}
E_{dAb}	436	kJ mol^{-1}
$k_{dAa\_52°C}$	0.0031	min^{-1}
$k_{dAb\_52°C}$	0.0005	min^{-1}
A_a	0.7988	
A_b	0.2012	
R^2	0.908	

Two models were used to describe the kinetic of cathepsin B and L activation and deactivation during *sous vide* cooking. The result of nonlinear least-squares fitting of both models were illustrated in Figure 3.4 and the obtained parameters value were presented in Table 3.2. Parameter $[I]_0$ (cathepsin B and L inside the lysosome) estimated from Model 1 was 3.67 U.g^{-1}meat. Activation energy of deactivation reaction (E_a=293 kJ mol^{-1}) was slightly higher compared to that of activation reaction (E_d=287 kJ mol^{-1}) which suggest that the deactivation reaction (denaturation of active cathepsin B and L) was more temperature dependent compared to the activation reaction (release of active cathepsin B and L from the lysosome). The result of coefficient of determination shows that Model 1 can satisfactorily describe the experimental data (R^2=0.900) (Table 3.2). However, it is apparent from Figure 3.4 that Model 1 cause overprediction of the deactivation reaction especially in the low-temperature treatment such as 48, 50, 52, 54, and 56°C. In this temperature range, the experimental data shows two deactivation phases. At the first few hours, experimental data shows deactivation curve following Model 1. However, at a longer heating time, the deactivation curve deviates from Model 1 and seems plateau. The existence of two

deactivation phase suggests the presence of two isoenzymes with different thermal resistances (Liing & Lund, 1978). The presence of two enzymes is expected since the substrate used is a common substrate for two cathepsins which are cathepsin B and cathepsin L.

Model 2 assume the existence of two isoenzymes with different thermal stability, which each deactivated following a first-order reaction. A better fit was found with Model 2 (R^2=0.908) compared to Model 1. The value of $[I]_0$ and activation parameters (E_a and $k_{a\_52°C}$) were comparable between the two models. Additionally, the results reveal that the thermally stable enzyme fraction (A_b), characterized by a smaller deactivation rate constant ($k_{dAb\_52°C}$=0.0005 min^{-1}) compared to the thermally labile fraction ($k_{dAa\_52°C}$=0.0031 min^{-1}), constitutes 20.1 % of the cathepsin B and L activity, while the thermally labile enzyme fraction accounts for 79.9 % of the cathepsin B and L activity. Furthermore, the thermally stable fraction exhibits a higher activation energy of deactivation (E_{dAb}=436 kJ mol^{-1}) compared to the labile fraction (E_{dAa}=270 kJ mol^{-1}), indicating their pronounced temperature dependencies. From Figure 3.5 it can be seen the thermal-labile fraction can be associated with cathepsin B while the thermal-stable fraction can be associated with cathepsin L. This is in agreement with the result reported by Wang et al. (2013) and Kaur et al. (2020), which found that cathepsin B is more thermally labile compared to cathepsin L.

The values of kinetic parameters obtained in this study are higher than the values obtained by Li et al. (2021) for cathepsin B activity in the sarcoplasmic extract of yellow-feathered chicken. This difference might be due to the presence of other proteins or cellular components that affect the thermal stability of cathepsin B in intact meat compared to the sarcoplasmic extract.

Even though the result shows that Model 2 describes the experimental data the best, Model 2 only gave a slight increase in the R^2 value compared to Model 1. In addition, Model 2 is more complex because it contains eight fitting parameters compared to Model 1 which only have five. As a result, Model 1 was used for subsequent data analysis.

Time to reach maximum (t_{max}) of cathepsin B and L activity during *sous vide* cooking at different temperature is presented in Table 3.3. The maximum cathepsin B and L activity ($[A]_{max}$) calculated in this study was 4.85 $U.g^{-1}$meat. Since the rate of cathepsin B and L activation

increased with increasing temperature, the maximum activity of cathepsin B and L was reached earlier at higher cooking temperatures. At 60 °C, maximum cathepsin B and L activity was obtained as early as 30 seconds after start of cooking. While at 50 °C, the maximum activity was achieved after 13.7 minutes cooking. Overall, the time to reach maximum cathepsin B and L activity was less than one hour in the temperature range used in this study (46 – 60 °C).

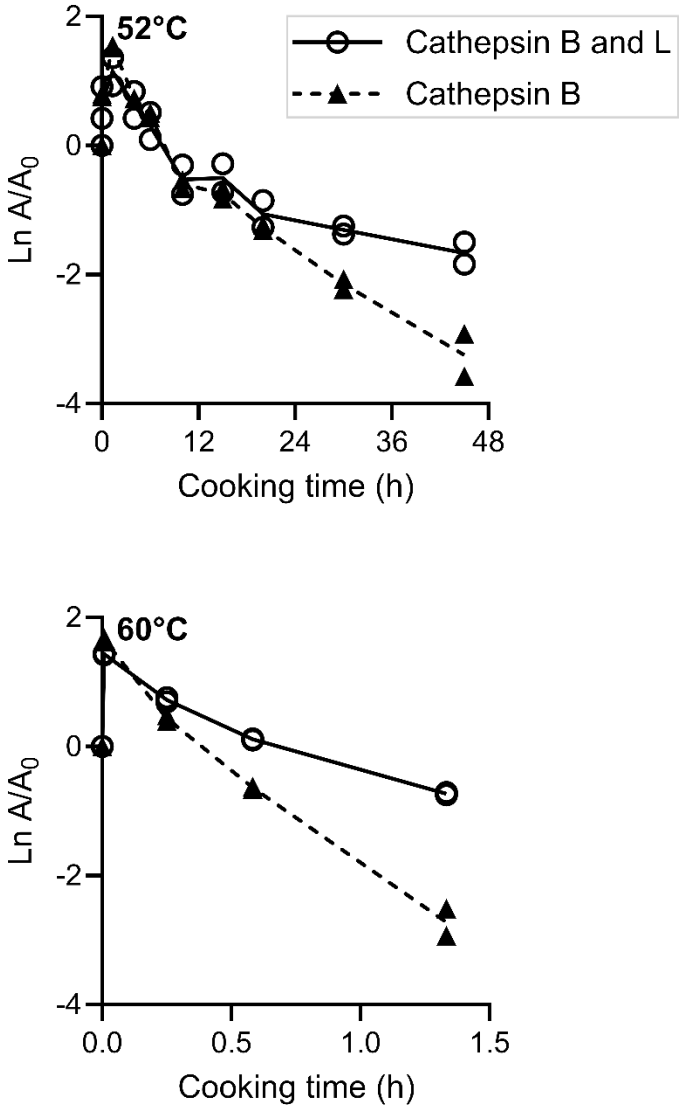

Figure 3. 5 Comparison between cathepsin B and cathepsin B and L activity in beef brisket *sous vide* cooked at 52 and 60 °C. The symbol shows individual value while the line shows the average value of the experimental data.

Table 3. 3 Time to reach maximum cathepsin B and L activity (t_{max}) during *sous vide* cooking at different temperatures based on Model 1.

Temperature (°C)	t_{max} (min)
46	52.4
48	26.7
50	13.7
52	7.1
54	3.7
56	1.9
58	1.0
60	0.5

Estimated parameters (Table 3.2) obtained in this study was based on the experimental data collected using meat from the same muscle cut (brisket), pH value (pH 5.6), and aging time (6 days post-mortem). As a consequence, the applicability of estimated parameters obtained in this study to other meat that have different properties should be further evaluated. Parameter $[I]_0$ (cathepsin B and L inside the lysosome) might be different for meat from different species (Etherington, Taylor, & Dransfield, 1987), muscle types (Kirchofer, Calkins, & Gwartney, 2002; Taylor, Almond, & Etherington, 1987), animal breed (Johnson, Calkins, Huffman, Johnson, & Hargrove, 1990), animal castration status (Wu, Dutson, Valin, Cross, & Smith, 1985), and meat aging time (Ertbjerg, Mielche, Larsen, & Møller, 1999; Wang et al., 2022). Etherington et al. (1987) reported that the level of cathepsin B and L was highest in pork, followed by rabbit, calf, lamb, chicken, and lowest in beef where cathepsin B and L concentration in pork was 4.3-fold higher than in beef. Furthermore, since the value of $[I]_0$ is related to the value of $[A]_0$ (initial cathepsin B and L activity that has been released in raw meat), aging time might affect the parameter $[I]_0$. Higher cathepsin B and L concentration in soluble fraction of beef *pectoralis profundus* was found after 7 days aging compared to 1 day while no significance difference was found between 7 and 21 days (Ertbjerg, Mielche, et al., 1999). Similar result was reported by (Wang et al., 2022) that found significance increase in cathepsin B and L activity with increasing aging time (2, 6, and 10 days post-mortem) in beef *semitendinosus* muscle.

Besides, estimated parameters also might be different for meat with different pH value than was used in this study. Low pH can destabilize lysosomal membrane thus facilitating the release of cathepsin B and L into sarcoplasm. Ertbjerg, Mielche, et al. (1999) reported an increase in cathepsin B and L activity with a decrease in meat pH value by lactic acid injection. Similarly, Lomiwes, Farouk, Wu, and Young (2014) found that higher concentration of active cathepsin B activity in sarcoplasmic of low pH meat (≤ 5.79) compared to high pH meat ($pH \geq 6.2$) which suggest greater stability of lysosomal membrane at higher pH. Due to this, it can be hypothesised that during heating, meat with high pH value might have a lower rate of cathepsin B and L activation compared to those with low pH value.

Muscle pH is also known to affect the thermal stability of enzymes. Okitani, Matsukura, Kato, and Fujimaki (1980) demonstrated that purified cathepsin L was stable when heating at temperature 37 °C for 1 h at pH 5.0, 5.2, and 5.5. However, its loss almost 25, 30, and 70 % of its activity under the same heat treatment at pH 6.0, 6.5, and 7.0, respectively. The decrease in cathepsin thermal stability at pH higher than 5.5 also has been reported by Mason, Green, and Barrett (1985) and Dufour, Dive, and Toma (1988). In addition, high salt content also decreases thermal stability of cathepsin B and L. Zhao et al. (2005) reported that an increase in salt content from 0 to 10 % caused a decrease in cathepsin B and L activity in Jinhua ham with higher decrease was observed with increasing temperature from 4 to 40 °C. Thus, meat with high pH value and salt content (brine) might have a higher thermal deactivation rate compared to those with normal pH and salt content. However, this requires further investigation since meat is a complex system in which high pH also increases the stability of other proteins such as small heat shock proteins (sSHP) that might affect the thermal stability of cathepsin B and L in intact meat during heating (Lomiwes et al., 2013).

3.3.2 Effect of temperature on the rate of reaction of cathepsin B and L

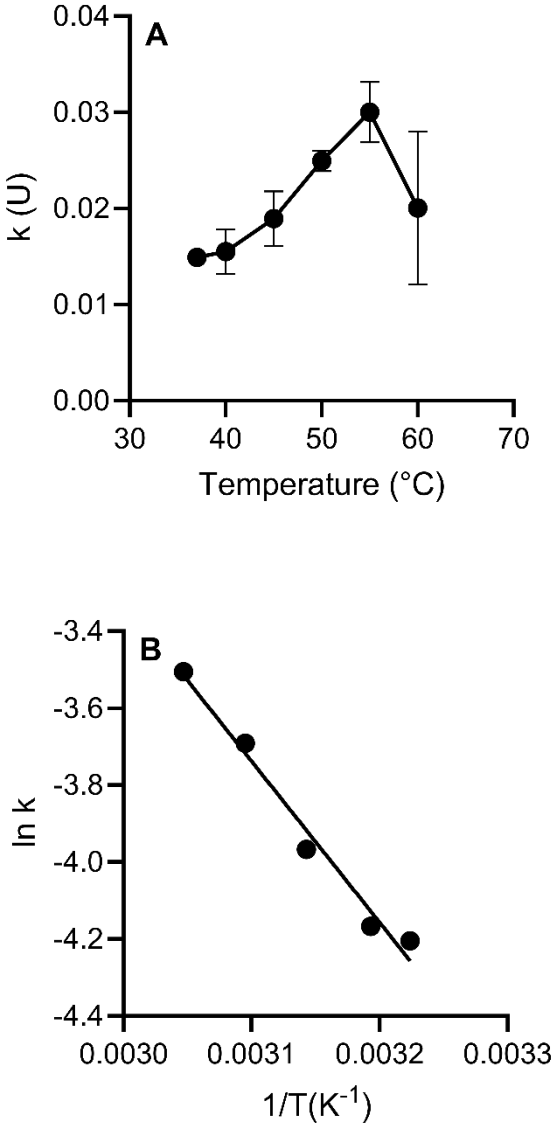

Figure 3. 6 A. Effects of temperature on cathepsin B and L rate of reaction (k) (Error bar indicates standard deviation, n=3). **B.** Arrhenius plot for the temperature dependence of cathepsin B and L rate of reaction. 1 U is equal to 1 nmole AMC released per min at that specified temperature.

The results in the previous section describe the effect of temperature and time on the cathepsin B and L activity. However, it is known as 60 °C, the cumulative activity temperature also influences the rate of enzyme reactions. In order to determine the potential of cathepsin B and L in

modulating tenderisation during *sous vide* cooking through proteolytic activity, the effect of temperature on the rate of reaction should also be considered. Figure 3.6A shows the effect of temperature on the rate of reaction of cathepsin B and L towards synthetic substrate (Z-Phe-Arg AMC) at pH 5.5. The graph shows that the rate of reaction increases as temperature increase and reaches maximum value at 55 °C before it decreases at higher temperatures due to enzyme deactivation. Thus, from the data measured at temperature between 37 and 55 °C, an Arrhenius plot was constructed (Figure 3.6B). Cathepsin B and L activation energy of 35 kJ.mol^{-1} ± 2.87 was obtained from the slope of Arrhenius plot (R^2=0.98).

3.3.3 Cumulative cathepsin B and L activity during *sous vide* cooking.

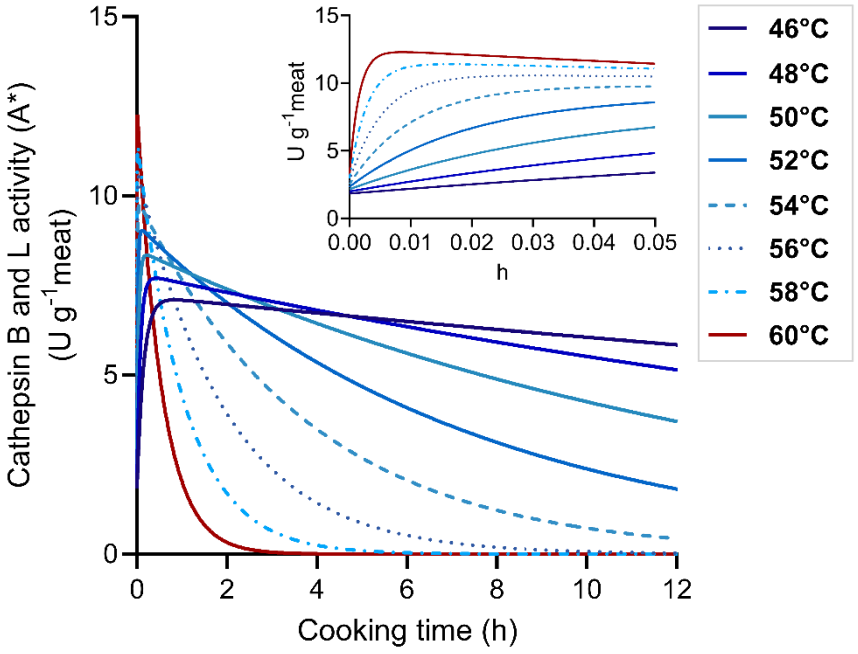

Figure 3. 7 Cathepsin B and L activity (A*) during *sous vide* cooking obtained after including the effect of temperature on both cathepsin B and L activity (predicted from Model 1) and rate of reaction. The inset depicts the activation phase.

Figure 3.7 presents the cathepsin B and L activity (A*) obtained after including the effect of temperature on cathepsin B and L activity and rate of reaction. It is apparent from Figure 3.6 that the highest cathepsin B and L activity (A*) can be observed at 60 °C. This is due to higher rate

of enzyme reaction at higher temperature compared to the lower temperatures. However, as can be expected, this high cathepsin B and L activity (A*) can only be observed for a short time due to the high deactivation rate at this temperature.

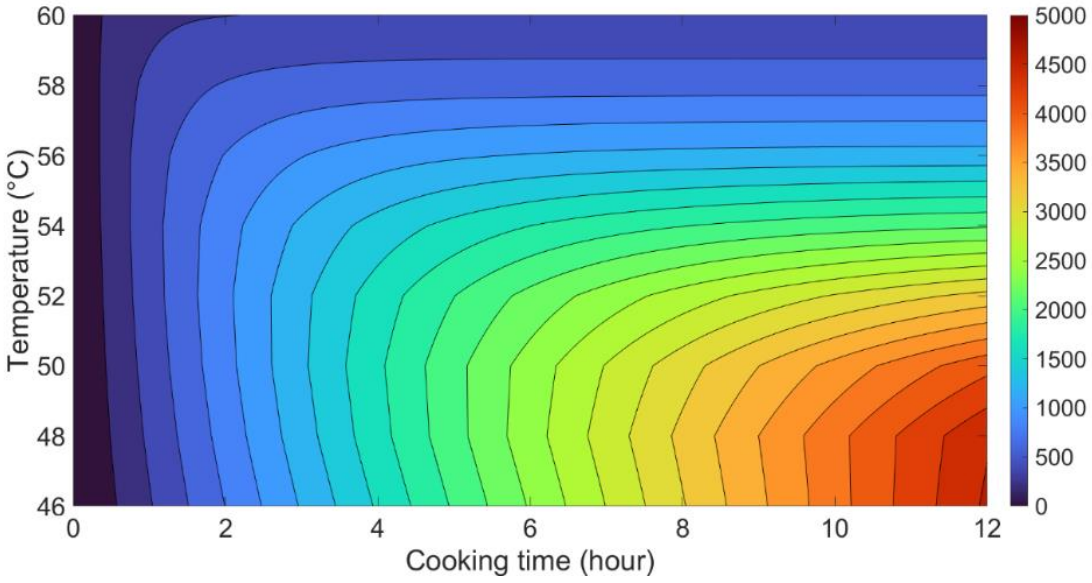

Figure 3. 8 Contour plot showing cumulative cathepsin B and L activity during 12-hour *sous vide* cooking. Each contour represents 200 cumulative cathepsin B and L activities (nmoles AMC g^{-1}meat). The side colour bar serves as the legends.

The area under the curve of cathepsin B and L activity (A*) was used to determine the cumulative cathepsin B and L activity at specific temperature and time during *sous vide* cooking. This cumulative activity reflects the total amount of protein degradation that can occurred over the specified temperature and time interval. The obtained result was visualized using contour plot (Figure 3.8). It is apparent from the contour plot that the cumulative cathepsin B and L activity continuously increases with increasing heating time in low-temperature range as can be seen with continuous change in contour colour. For example, high cumulative cathepsin B and L activity (about 5000 nmoles AMC g^{-1}meat) can be achieved when cooking at 46 °C for 12 h. This is simply due to the slower rate of deactivation at this temperature allowed the residual active enzyme to continuously degrade meat proteins thus contribute to the increasing amount of proteolysis over time. In contrast, in high-temperature range such as 60°C, the cumulative activity

becomes constant (400 nmoles AMC g^{-1}meat) at a shorter cooking time due to the rapid rate of cathepsin B and L deactivation. There is no additional cumulative enzyme activity of *sous vide* cooking more than 2 h at 60 °C.

Additionally, since increasing the cooking temperature also cause an increase in activation rate and rate of enzyme reaction, there are certain time ranges where cooking at higher temperature produces greater cumulative activity compared to cooking at lower temperature, as can be seen by the contour line that was inclined to the left side (temperature axis) (Figure 3.8). This time ranges and the temperature to get the maximal cumulative activity is demonstrated in Figure 3.9 and their value of cumulative activity are presented in Table 3.4. This data can be used as a guidance to select temperature to get maximum cumulative cathepsin B and L activity when time is taken as the determining factor. For example, for a short time (10 minute and less), cooking at 60 °C give the highest cumulative cathepsin B and L activity. The temperature to achieve maximum cumulative cathepsin B and L activity then changes to lower temperatures as the time increase. For time more than 10.4 h, cooking at 46 °C give the highest cumulative cathepsin B and L activity.

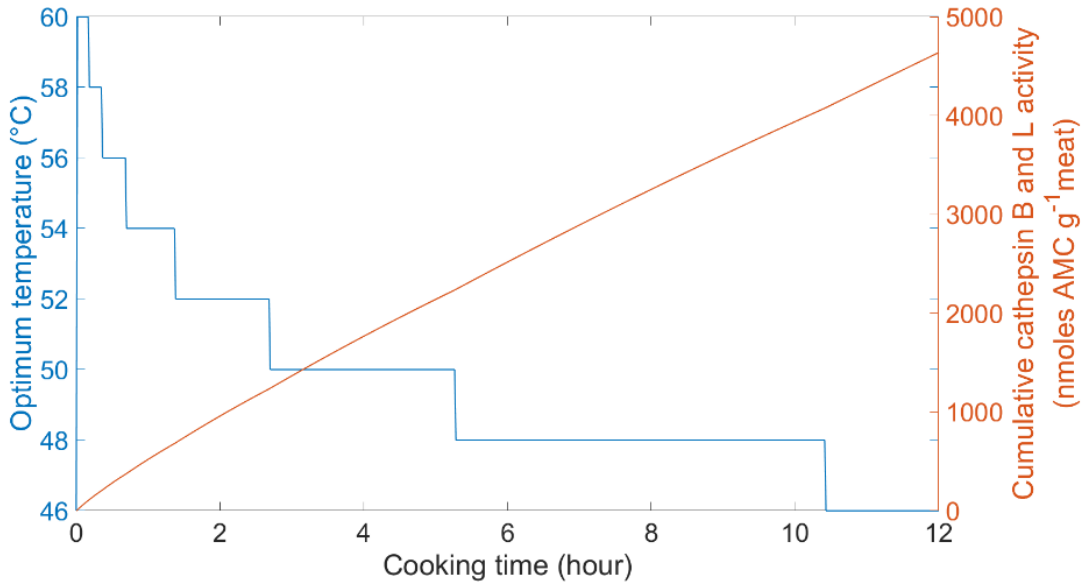

Figure 3. 9 Optimum temperature for maximum cumulative cathepsin B and L activity.

Table 3. 4 Optimum temperature for maximum cumulative cathepsin B and L activity at specific time ranges.

Cooking time	Optimum temperature (°C)	Maximum cumulative activity[a]
10min and less	60	103.3
>10min to 21min	58	202.6
>21min to 41min	56	368.4
>41min to 1.4h	54	679.9
>1.4h to 2.7h	52	1232.8
>2.7h to 5.3h	50	2233.7
>5.3 to 10.4h	48	4074.1
>10.4h	46	>4074.1

[a]Maximum cumulative activity (maximum cumulative cathepsin B and L activity in nmoles AMC g^{-1}meat).

3.4 Conclusion

Kinetics of cathepsin B and L activity during *sous vide* cooking has been successfully modelled following consecutive reactions with activation followed by deactivation. A better fit of experimental data was obtained with Model 2 (R^2=0.908) that assumes the existence of two isoenzymes with different thermal stability compared to Model 1 (R^2=0.900) which assumes single thermal stability fraction. Although Model 2 offered a better fit, the simplicity and acceptable R^2 value of Model 1 justified its use in predicting cathepsin B and L activity during *sous vide* cooking in the temperature range used in this study (46 – 60 °C).

The result shows that cooking temperature and time can be used to manipulate cumulative cathepsin B and L activity during *sous vide* cooking. Cooking at high temperature treatments can produce faster maximal cumulative cathepsin B and L activity, but only to a limited amount due to the faster rate of cathepsin B and L deactivation. On the other hand, cooking at low temperature treatment decrease the rate of cathepsin B and L deactivation thus allowing for high accumulation of enzyme activity over time. Further study is warranted to investigate whether high cumulative cathepsin B and L activity can translate into greater tenderisation. Thus, the effect of cooking at two different temperatures which are 46 °C and 56 °C that represent two different levels of cumulative cathepsin B and L activity on the tenderness of beef brisket was investigated in Chapter 4.

CHAPTER 4 Effect of *sous vide* cooking at different time-temperature combinations and levels of cumulative cathepsin B and L activity on meat tenderness

4.1 Introduction

Sous vide is a process of cooking foods inside heat-stable vacuum-sealed pouches under precise, controlled conditions of temperature and time (Baldwin, 2012). This cooking technique has gained interest in restaurants, home, and the food industry due to a greater consistency of the end products and its ability to tenderise tough meat cuts (Baldwin, 2012; Christensen et al., 2011; Fareh, 2018; Gámbaro et al., 2023; Naqvi et al., 2021).

Several studies have indicated the role of cathepsin B and L in meat tenderisation during *sous vide* cooking through proteolysis of myofibrillar proteins, also known as accelerated aging of myofibrillar proteins (Beilken, Bouton, & Harris, 1986; Christensen et al., 2013; Davey & Gilbert, 1976; Uttaro et al., 2019). The proteolysis of myofibrillar proteins weakens the myofibrillar structure, prevents the formation of large aggregates, and cause reduction in myofibrillar shrinkage, leading to tender meat (Dominguez-Hernandez & Ertbjerg, 2021; Huff-Lonergan et al., 2010; Yao et al., 2023). Besides, cathepsin B and L can degrade connective tissue protein and proteoglycan/ glycosaminoglycans, thus weakens the connective tissue structure and promotes collagen solubilisation, consequently, contributes to tenderisation of meat (Burleigh et al., 1974; P. Panwar et al., 2013). The previous chapter has shown the ability to obtain different cumulative cathepsin B and L activities by manipulating temperature and time of *sous vide* cooking. *Sous vide* cooking at low temperatures such as 46 °C causes a slower rate of cathepsin B and L deactivation thus allowing for the accumulation of enzymes activity over time. On the other hand, cooking at high temperatures such as 56 °C causes a faster rate of cathepsin B and L deactivation thus limiting cumulative enzymes activity. Thus, it is hypothesised that *sous vide* cooking at 46 °C for a prolonged time may produce greater tenderisation than at 56 °C. Accordingly, the objective of this study is to determine the degree of meat tenderisation that can

be achieved when *sous vide* cooking at 46 and 56 °C for prolonged time that represent high and low cumulative cathepsin B and L activity, respectively.

4.2 Materials and methods

4.2.1 Sample

Six hot-boned briskets from three beef carcasses (Hereford-Friesian cross heifers) were collected from an abattoir (ANZCO Foods Manawatu) approximately five hours after slaughter. The briskets were transported to the laboratory at Massey University in Palmerston North (30-minute drive), vacuum-packed, and stored at 4 °C until 72 h post-mortem.

4.2.2 Sample preparation

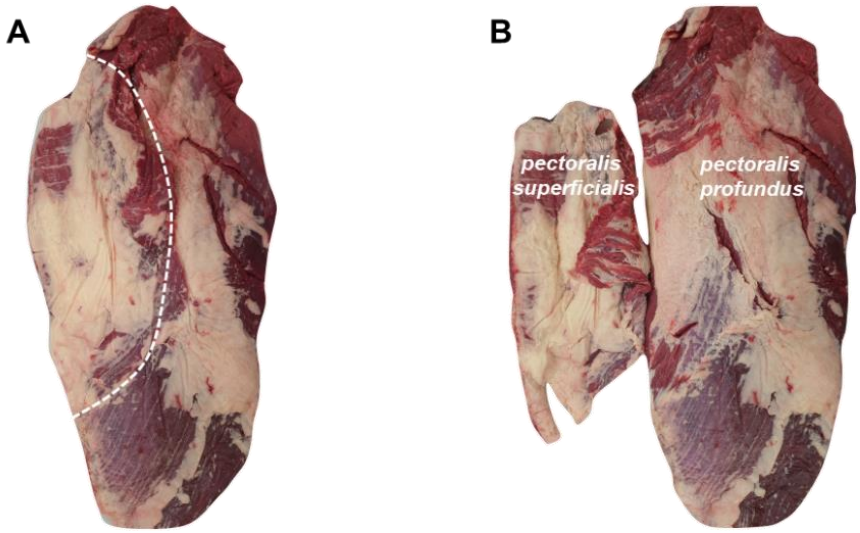

Figure 4. 1 Beef brisket: **A.** The dashed white line shows dissection point to remove *pectoralis superficialis* muscle from the brisket. **B.** The *pectoralis superficialis* muscle was discarded and the *pectoralis profundus* muscle was used for the study.

Only *pectoralis profundus* muscles of the briskets were used in this study. The *pectoralis profundus* muscle was prepared by removing *pectoralis superficialis* muscle from the briskets (Figure 4.1) (Handbook of Australian meat, 1998). In order to reduce variability within the briskets and to obtain steaks with uniform size, only the middle part of the brisket was used: the head, tail, and left and right sides were discarded from the brisket. The discarded portion was used for pH measurement. Subsequently, the briskets were cut across the muscle fibres into ten steaks with 2.5 cm thickness. The external fat and connective tissue were trimmed off from each steak.

The steaks were placed inside heat-stable vacuum bag (EVB vacuum bag, 95 µm thick), vacuum-packed to 15 mbar using a vacuum packing machine (Multivac New Zealand Ltd), weighed, and stored at 4 °C (Figure 4.2). Within each brisket, the first steak was used for raw meat analysis (total collagen, cathepsin B and L activity, and differential scanning calorimetry), while the rest of the sample were assigned for different heat treatments.

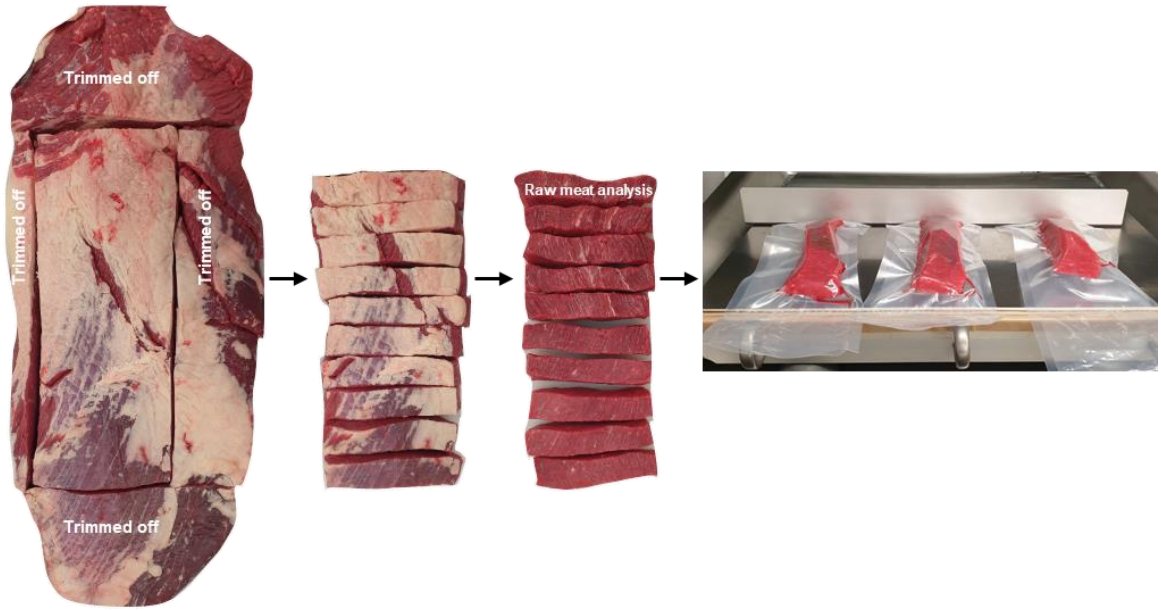

Figure 4. 2 The scheme of sample preparation which includes the removal of the head, tail, and left and right side of the brisket, follow by cutting the brisket across the muscle fibres into 10 steaks (2.5 cm thickness), then removal of external fat and connective tissue, and finally vacuum packing of the meat sample.

4.2.3 Further sampling

In the present study, each steak was further cut into two sections: a large one (3 × 10 × 2.5 cm, average 100 g) and a small one (3 × 2.5 × 2.5 cm, average 30 g) (Figure 4.3), that would be used for different treatments (see details below). All the samples were individually vacuum-packed, weighed, and stored at 4 °C.

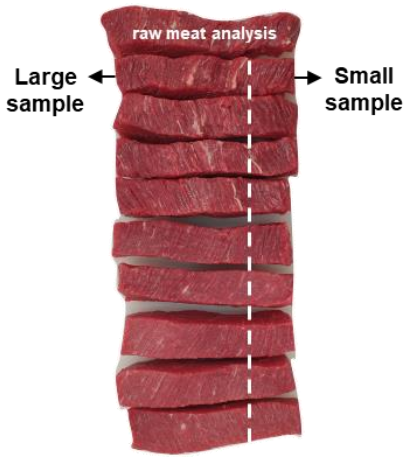

Figure 4. 3 Schematic diagram of *pectoralis profundus* sampling. The dashed white line shows the cut that divides each steak into the large and small samples.

4.2.4 Experimental design

The samples of brisket from the right side of carcasses were randomly assigned to one of 9 treatments: 46 °C over 8 different time periods (0, 12, 24, 36, 48, 60, 72, and 84 h) and one at 71 °C cooking (for standard tenderness evaluation) (AMSA, 2016). The samples of brisket from the left side of carcasses were randomly assigned to one of 9 treatments: 56 °C over 8 different time periods (0, 6, 12, 24, 36, 48, 60, and 72 h) and one at 71 °C cooking. The total of 18 treatment combinations were assigned to each individual animal (n=3). The samples were removed from the chiller and *sous vide* cooked at 4 days post-mortem. For 71 °C cooking, the samples were *sous vide* cooked at 71 °C water bath until the core temperature reached 71 °C. The large and its corresponding small sample were *sous vide* cooked at the same designated heat treatment. For 0 h treatment at 46 and 56 °C, the samples were *sous vide* cooked at the designated temperature until the core reached the temperature (no holding time). While for the rest of cooking time treatments, once the core temperature reached the designated temperature, the sample was held at that temperature for 6, 12, 24, 36, 48, 60, or 72 h. Once finished, the large samples were immediately transferred to 71 °C water bath until the core temperature reached 71 °C. Cooking was stopped by immersing samples in ice water baths for 15 mins. The large samples were used for shear force, cooking loss, and collagen measurements, while the small samples were used for

cathepsin B and L activity, differential scanning calorimetry (DSC), cooking loss, and collagen measurements. Sample for collagen and DSC analysis were stored in -20 °C until further analysis (Ngapo, Babare, Reynolds, & Mawson, 1999). Meanwhile, samples for cathepsin B and L extraction and shear force analysis were stored at 4 °C and extracted and analysed, respectively, within 24 h.

4.2.5 Cooking treatment

Figure 4. 4 Metal racks were used in the water bath to ensure uniform heating during cooking.

The samples were arranged in metal racks to avoid samples from overlapping and ensure uniform heating during cooking (Figure 4.4). The racks then were immersed in temperature-equilibrated circulating water bath according to the designated heat treatments. Since more than one water bath were used in this study, temperature on each water bath was standardized using a calibrated thermocouple. The cooking time was applied once the core temperature (Tc) of the steak reached the temperature of the water bath. Based on the preliminary study using a temperature data logger (Maxim's iButtons®, with temperature accuracy of ± 0.5 °C) placed in the geometrical centre of meat samples (large meat sample only), the time for Tc to reach 71 °C (temperature of water bath) was found to be 30 minutes (Figure 4.5). Thus, the time for Tc to reach any temperature treatment from initial temperature of 4 °C was fixed at 30 minutes (Gurney & Lurie, 1923).

The temperature-time profile of the meat sample was also modelled. The meat sample was assumed to be a slab with infinite length. Sample thickness and height were 25 mm and 30 mm, respectively. Heat transfer by conduction inside the meat sample was modelled using two dimension (2D) and the first kind of boundary condition approach, Equation (4.1). The value of thermal properties of meat were used according to the value reported by Oillic, Lemoine, Gros, and Kondjoyan (2011) which are 0.45 W m^{-2} °C^{-1}, 1060 kg m^{-3}, and 3200 J kg °C^{-1} for meat thermal conductivity, density, and thermal capacity, respectively.

$$\boldsymbol{Y_{av} = \frac{8}{\pi^2}\sum_{m=0}^{\infty}\frac{1}{(2m+1)^2}\,exp\left[-(2m+1)^2\frac{\pi^2}{4}Fo\right]} \quad \textbf{(4.1)}$$

$$\boldsymbol{Fo = \frac{\lambda t}{pcR^2}}$$

Where;

$Y_{av} = (\theta_{av} - \theta_a)/ (\theta_i - \theta_a)$

t = time (sec)

θ_{av} = average temperature in meat (°C)

θ_a = water bath temperature (°C)

θ_i = meat initial tempearture (°C)

R = meat half thickness (m)

λ = meat thermal conductivity (W m^{-2} °C^{-1})

c = meat thermal capacity (J kg °C^{-1})

p = meat density (kg m^{-3})

Figure 4.5 shows the calculated temperatures and the temperature measured at the centre of the meat sample using a Maxim's iButtons®. The calculated temperature required a longer time to reach 71 °C compared to measured temperature which was 37 minutes compared to 30 minutes, respectively. The time for the temperatures of the meat sample to reach 71 °C from initial temperature of 46 and 56 °C was also calculated. The result shows that the meat sample requires

30 and 32 minutes to reach the end temperature of 71 °C from initial temperature of 46 and 56 °C, respectively.

In the present study, the time for Tc to reach any temperature treatment from initial temperature of 4 °C and to reach the end temperature of 71 °C from initial temperature of 46 and 56 °C was fixed to 30 minutes. This was justified since based on the calculation the temperatures of the meat sample already reached more than 99 % of the end temperature after 30 minutes of heating at any condition mentioned above.

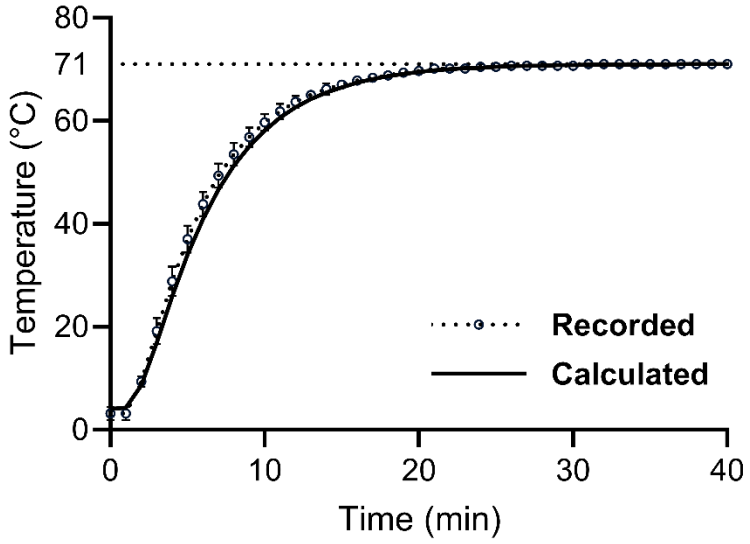

Figure 4. 5 Temperature profile at geometrical centre (Tc) of large meat samples recorded using Maxim's iButtons® temperature data logger and calculated using two dimension (2D) and the first kind of boundary condition approach. Mean ± standard deviation (n = 3).

4.2.6 pH measurement

The pH value of the meat samples was measured using a pH meter (Orion 3 Star pH Benchtop, Thermo Electron Corporation) equipped with a glass electrode and a temperature sensor. Prior to measurement, the pH meter was calibrated with pH 4 and pH 7 standard solutions. The meat pH measurement was done by inserting the glass electrode and temperature sensor into the centre of the sample. Two measurements were made for each brisket (Korkeala et al., 1986).

4.2.7 Shear force measurement

Square cross-section cores (10 × 10) (Silva et al., 2015) were removed parallel to the muscle fibre using a two-bladed knife as a guide and a sharp knife to cut the sample. Four to six cores were obtained for each meat sample. Shear force measurements were performed using texture analyser (Model TA-XTPlus, Stable Micro Systems Ltd, UK) with 50 kg load cell and triangular cutting (V-shaped) blade attachment (1.1684 mm thickness). The central portion of the sample was positioned under the blade and sheared perpendicular to the muscle fibre direction with crosshead speed 200 mm/min (AMSA, 2016). The maximum force required to cut through the samples was recorded as peak shear force value which was expressed in Newtons.

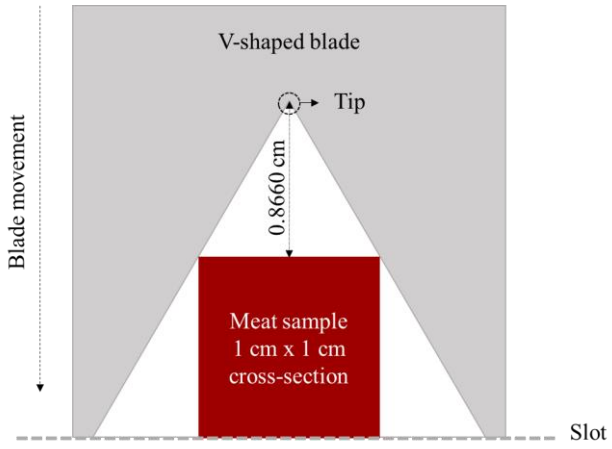

Figure 4. 6 V-shaped (60° angle) cutting blade position relative to the slot setting for the shear force measurement.

From the shear force measurement, the shear force deformation curve was also qualitatively analysed to illustrate the changes in the initial yield forces, final yield forces, and the shape of the curve. The initial yield force corresponds to the force required to initiate fracture, which is also evident as an inflection point in the curve (Bouton, Harris, & Shorthose, 1975). In this study it occurred at approximately 0.866 to 1 cm of blade travel distance. Whereas the final yield force was defined as the force occurring at the position where the blade had completely cut through the meat sample (Møller, 1981). In this study, it occurred approximately at 1.866 cm of blade travel

distance. Figure 4.6 illustrates the position of the cutting blade to the slot setting during the shear force measurement.

4.2.8 Cooking loss

Cooked meat samples were blotted dry and weighed. The amount of cook loss was calculated as below:

$$\boldsymbol{Cook\ loss}\ (\%\ \boldsymbol{w/w}) = \frac{\boldsymbol{W_0\ (g) - W_c\ (g)}}{\boldsymbol{W_0\ (g)}}\ \boldsymbol{x\ 100} \qquad \textbf{(4.2)}$$

Where;

W_0 = weight of meat before cooking

W_c = weight of meat after cooking.

4.2.9 Residual cathepsin B and L activity

Enzyme extraction was carried out as described in Chapter 3, section 3.2.4 except in the present study enzyme extraction was performed separately for the meat residue and the cook loss fraction. Cook loss was filtered using a 0.45 µm syringe filter and aliquoted into 1.5 ml Eppendorf tubes and immediately stored at -20 °C until further analysis. Cathepsin B and L activity was performed as described in Chapter 3, section 3.2.5, 3.2.7, and 3.2.8.

4.2.10 Differential scanning calorimetry (DSC) analysis

Thermal denaturation properties of raw and cooked meat were analysed using DSC Q2000 V24.11 Build 124 (TA Instruments, New Castle, DE, USA) fitted with intercooler unit with refrigerating cooling system (RCS). Nitrogen gas was used as purge gas at 50 ml min^{-1} and the DSC was calibrated using indium and water at 5 °C min^{-1}. A subsample of 20 mg was dissected from meat sample and weighed into Tzero aluminium hermetic pans, and then hermetically sealed using Tzero press. The weight of sample and the total weight of pan was recorded. An empty sample pan was used as a reference. The heating was performed with 1 min equilibration at 25 °C followed by heating step at 5 °C min^{-1} to 90 °C and cooling step at 30 °C min^{-1} to 25 °C. After

measurement, the pan was weighed again to ensure that no water loss occur during the measurement. The pan then was punctured with a needle, dried at 106 °C in an oven for 24 h, cooled in a desiccator for 1 h, and weighed to determine sample dry weight.

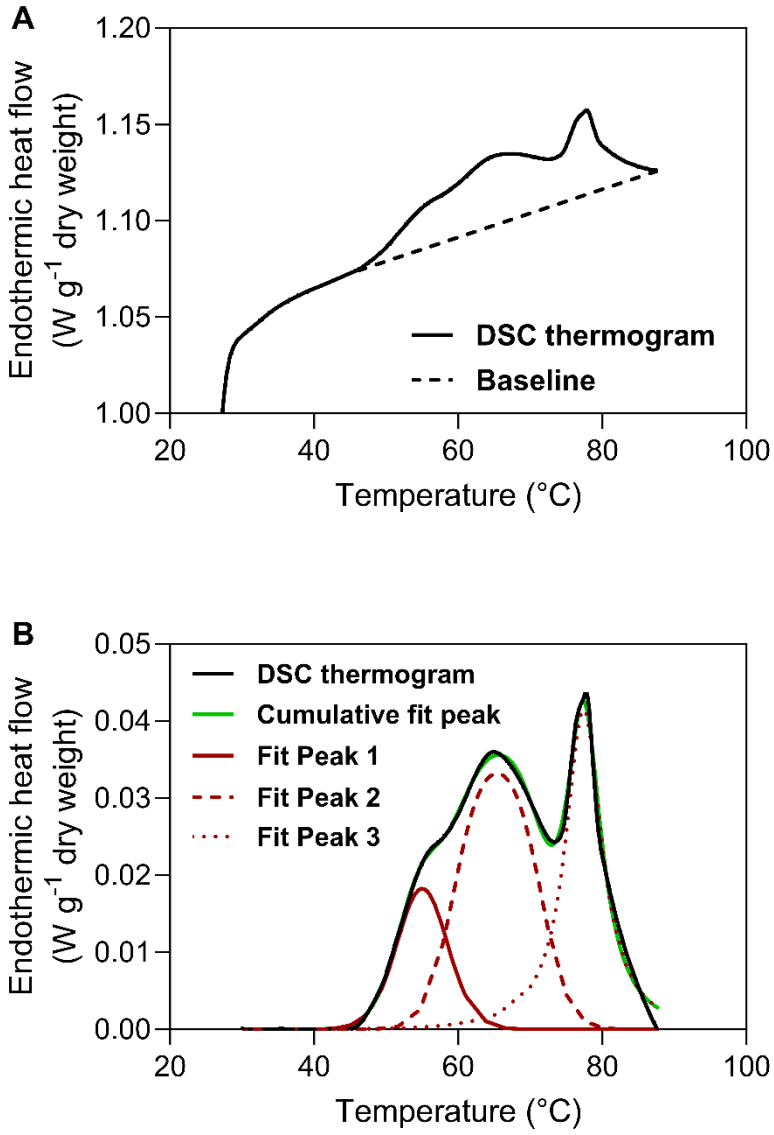

Figure 4. 7 A. DSC thermogram of raw beef brisket at heating rate of 5 °C min^{-1} and constructed baseline. **B.** Deconvolution result of raw beef brisket DSC thermogram using ©OriginPro software, DSC thermogram separated as three main peaks.

DSC thermograms were analysed using ©OriginPro v9.9.0.220. First, a baseline connecting beginning and end points of the endothermic heat flow data by a straight line was constructed

(Figure 4.7A). The points were defined manually. The starting and end point were defined at 45 °C and 87.9 °C, respectively.

Peak-fitting analysis was carried out using Gaussian-Lorentzian Cross function to deconvolute DSC endotherms into three individual peaks which are Peak 1, Peak 2, and Peak 3 that refer to the first, second, and third peak in the DSC thermogram, respectively. The initial position of the peak centre (T_{max}) for individual peak was assigned manually. The baseline was fixed, but other fitting parameters such as peak centre, amplitude, width, and shape was allowed to change during the fitting. The result of deconvolution was presented on Figure 4.7B. Area under individual peak obtained from the fitting was used to calculate the enthalpy of denaturation (ΔH) based on sample dry weight (Equation 4.3).

$$\boldsymbol{Enthalpy\ of\ denaturation, \Delta H\ (J\ g^{-1} dry\ weight)} = \frac{\boldsymbol{Area\ under\ individual\ peak\ (mJ.sec^{-1}.℃)}}{\boldsymbol{Sample\ dry\ weight\ after\ DSC\ (mg) x DSC\ heating\ rate\ (℃.sec^{-1})}} \quad \textbf{(4.3)}$$

4.2.11 Collagen determination

Collagen was determined following method as described by Latorre, Velázquez, and Purslow (2018a) with slight modifications. For total collagen, raw meat sample was used. For cooked meat sample, contents of the vacuum bags were separated into the meat residue and cook loss fractions, and the collagen content was assayed in each fraction. Raw meat, meat residue (sliced thinly) and cooking loss were stored in -20 °C until further analysis. On the day of hydrolysis, the meat sample (not thawed) was pulverised using multipurpose grinder (Model BCG200, Breville Pty Ltd, Australia). While the cook loss was thawed and homogenized. The meat sample (1 g) and cook loss (2 g) were weighed individually into 25 ml *Kimax* glass tube with screw cap and then hydrolyzed in 5 ml HCl (6 N) at 110 °C for 16 h. The higher weigh of sample for cook loss fraction is due to low concentration of collagen in this fraction. After cooled down, 5 ml freshly prepared sodium hydroxide (NaOH, 6 M) was added into the hydrolysates. The mixtures then filtered using Whatman Filter Paper No 1 and transferred to 50 ml volumetric flask and total volume was filled up to 50 ml using deionize water. Each fraction was further diluted appropriately with deionized

water, so that the absorbance would fall within the linear range of standard hydroxyproline. Total dilution was 250 x and 50 x for meat sample and cook loss fraction, respectively.

The hydroxyproline concentration was determined following Bergman and Loxley (1963). Sample (100 µl), isopropanol (200 µl), and oxidant solution (100 µl) were pipetted into 2 ml microcentrifuge tube. The mixture was let to stand at room temperature for 4 min before 1.3 ml Ehrlich's reagents (1.3 ml) was added. The mixture was then vortexed and incubated at 60°C for 25 min in a water bath. The heating was stopped by cooling the mixtures in cold water for 2 – 3 min. The solution was transferred into 1 cm cuvette and the absorbance against water was measured at 558 nm using a UV-visible spectrophotometer (Evolution 201, Thermo Fisher Scientific Ltd., USA).

The collagen content was calculated by multiplying the concentration of hydroxyproline by 7.14, which assumes that the average concentration of hydroxyproline in collagen is 14% (Etherington & Sims, 1981). Total collagen is the value of collagen in raw meat. The values of solubilised collagen in cook loss (ColCL) and collagen in meat residue (ColMR) were expressed as the ratio between collagen content (mg) and weight of meat before cooking (g).

$$\boldsymbol{Total\ collagen\ (mg\ g^{-1}\ meat)} = \frac{\boldsymbol{Collagen\ (mg)}}{\boldsymbol{W_S(g)}} \quad \textbf{(4.4)}$$

$$\boldsymbol{ColCL\ (mg\ g^{-1} raw\ meat)} = \frac{\boldsymbol{Collagen\ per\ gram\ cook\ loss\ (mg\ g^{-1} cook\ loss)\ x\ W_{cl}(g)}}{\boldsymbol{W_0(g)}} \quad \textbf{(4.5)}$$

$$\boldsymbol{ColMF\ (mg\ g^{-1} cooked\ meat)} = \frac{\boldsymbol{(Total\ collagen - ColCL)\ x\ W_0}}{\boldsymbol{W_c\ (g)}} \quad \textbf{(4.6)}$$

$$\boldsymbol{SC}\ (\%) = \frac{\boldsymbol{ColCL}}{\boldsymbol{Total\ collagen}}\ \boldsymbol{x\ 100} \quad \textbf{(4.7)}$$

Where;

ColCL= solubilised collagen in cook loss

ColMF = collagen in meat residue

SC = solubilised collagen in cook loss per total collagen

W_S = weight of raw meat sample

W_0 = weight of meat before cooking

W_c = weight of meat after cooking

W_{cl} = weight of cook loss.

4.2.12 L-hydroxyproline standard curve

Standard solutions of L-hydroxyproline at the concentrations of 2, 4, 6, 8, 10 µg ml^{-1} were prepared from trans-4-hydroxy-L-proline (Sigma-Aldrich) using water as the diluent. Standard curves were performed following the method described in 4.2.7 with the L-hydroxyproline solution at a pre-defined concentration was used in place of the sample solution (100 µl). While blank was created by substituting the sample solution with deionized water. Figure 4.7 shows the standard curves of L-hydroxyproline. The slope of standard curves was calculated using linear regression (Microsoft® Excel®).

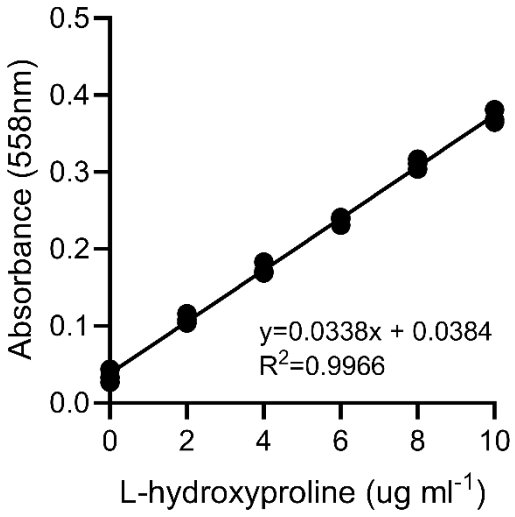

Figure 4. 8 Standard curves of L-hydroxyproline.

4.2.13 Residual cathepsin B and L model prediction and cumulative activity

Residual cathepsin B and L was predicted using Model 1 (Equation 3.6), Chapter 3, section 3.2.9.1. based on estimated parameters presented in Table 3.2 and initial concentration of

cathepsin B and L of 1.56 U g^{-1}meat obtained in the present study. Cumulative cathepsin B and L activity was determined based on method described in Chapter 3, section 3.2.11.

4.2.14 Statistical analysis

Statistical analysis was performed using SAS® Studio 3.8 (SAS Institute, Cary, NC, USA). All data were tested for homogeneity of variance using Levene's test. The SAS PROC MIXED procedure was used to evaluate the effect of heat treatments on cathepsin B and L in meat fraction, cathepsin B and L in cook loss, peak shear force, cooking loss, and SC. Temperature, time, and their interaction were specified as fixed effects, animal as random effect, and location within animal as repeated effect. When fixed effects were significant, multiple comparison were performed using the Tukey procedures ($P < 0.05$ indicated significance). Data that did not fulfil the assumption of equal variance were log (cathepsin B and L in meat residue and cathepsin B and L in cook loss) and square root transformed (solubilised collagen in cook loss per total collagen, SC) before analysed. The data were then back transformed to present the result. In addition, the SAS PROC CORR procedure was used to determine Pearson correlation coefficients between shear force values and other measured variables. All figures in this study were created using GraphPad Prism v9.4.0. The technical replicates were first averaged, and then the values were reported as the mean of three animal replicates (n=3) ± standard error of mean (SEM), unless specified otherwise.

4.3 Results

4.3.1 Beef brisket characteristics

Table 4. 1 Characteristics of raw and *sous vide* cooked beef brisket at 71 °C (T_C).

Attribute	Mean ± SEM
Raw brisket	
pH	5.48 ± 0.03
Total collagen (mg g^{-1} meat)	12.5 ± 2.93
Cathepsin B and L activity (U g^{-1} meat)	1.56 ± 0.55
DSC analysis[1]	
$T_{maxPeak1}$ (°C)	54.5 ± 0.32
$T_{maxPeak2}$ (°C)	65.2 ± 0.12
$T_{maxPeak3}$ (°C)	77.5 ± 0.07
ΔH_{0Peak1} (J g^{-1} dry weight)	1.47 ± 0.20
ΔH_{0Peak2} (J g^{-1} dry weight)	5.13 ± 0.40
ΔH_{0Peak3} (J g^{-1} dry weight)	3.50 ± 0.13
ΔH_{0total} (J g^{-1} dry weight)	10.10 ± 0.27
***Sous vide* cooked brisket at 71 °C**	
Peak shear force (N)	45.1 ± 5.97
Cooking loss (% w/w)	20.9 ± 2.05
Solubilised collagen in cook loss per total collagen (%)	0.74 ± 0.22

[1]DSC analysis was performed at heating rate of 5 °C min^{-1}.
Tc (the core temperature of meat reaches the applied temperature)

Table 4.1 shows the properties of raw and *sous vide* cooked beef brisket at 71 °C (T_C) used in the present study. The pH value of the raw meat is 5.48 which is in the range of normal post-rigor pH value of beef. The values obtained in this study are in the range of values reported for total collagen (Hammond et al., 2022; Latorre, Velázquez, & Purslow, 2018b), cathepsin B and L activity (Ertbjerg, Mielche, et al., 1999), and proteins thermal denaturation properties (Chian et al., 2021) of beef brisket. Beef brisket *sous vide* cooked at 71 °C (Tc) has a mean shear force value of 45.1 N. However, it is worth noting that the peak shear force value obtained in the present study could not be compared directly to the peak shear force value reported in consumer tenderness threshold studies due to the different gap clearance between blade and slot used for shear force measurement in the present study. The standard Warner-Bratzler shear blade and slot reported in tenderness threshold study (Destefanis, Brugiapaglia, Barge, & Dal Molin, 2008;

Holman, Collins, Kilgannon, & Hopkins, 2020; Huffman et al., 1996; Rodas-González, Huerta-Leidenz, Jerez-Timaure, & Miller, 2009) is 1.1684 mm of blade thickness and 2 mm of slot spacing as suggested by (AMSA, 2016). The blade thickness used in the present study has the same thickness but the slot spacing is higher which is 4 mm which bring about larger gap clearance between blade and slot. Voisey (1976) reported a decrease in shear force value with increasing gap clearance between blade and slot. Thus, the value of shear force reported here might be higher if the same setting was used as in tenderness threshold studies. The peak shear force value of <42.9 N (Destefanis et al., 2008), <42.6 (Holman et al., 2020), <40.1 (Rodas-González et al., 2009), and 40.2 N and less (Huffman et al., 1996) have been reported as consumer thresholds for tenderness ranking and acceptability. Overall, the beef brisket used in the present study can be considered a tough meat cut and does not meet the tenderness acceptability criteria for consumers.

Table 4. 2 Influence of *sous vide* cooking temperature, time, and temperature x time interaction on the properties of beef brisket.

Meat properties	Temperature	Time	Temperature x time
Residual cathepsin B and L in meat residue (U g^{-1} meat)	***	***	NS
Residual cathepsin B and L in cook loss (U g^{-1} meat)	***	***	*
ΔH_{Peak2}	***	***	NS
ΔH_{Peak3}	***	***	NS
Shear force (N)	***	***	***
Cooking loss (% w/w)	NS	***	NS
Solubilised collagen in cook loss per total collagen (%)	***	***	NS

NS = non-significant
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

4.3.2 Cathepsin B and L activity

Figure 4.9(A) and 4.9(B) shows the effect of *sous vide* cooking at 46 °C and 56 °C for 6 to 72 h on residual cathepsin B and L activity in meat residue and cook loss fraction, respectively. There was a significant effect of temperature and time on cathepsin B and L activity in meat residue

(Table 4.2) ($p < 0.001$). A significantly higher cathepsin B and L activity was found in meat sample cooked at 46 °C compared to 56 °C at all time treatment. At both cooking temperatures, cathepsin B and L activity decreased with increasing cooking time. Similarly, a significantly higher cathepsin B and L activity was observed in cook loss of meat cooked at 46 °C compared to 56 °C at all time treatments. It is worth noting that the cathepsin B and L activity in the cook loss fraction is approximately only 4 % of the meat residue fraction. This indicates that most of the cathepsin B and L that have been released from lysosomes into the cytosol upon heating, are retained within the meat, and only small proportion of cathepsin expelled to the surface of meat along with water.

Figure 4.10 shows the comparison between the experimental data of residual cathepsin B and L activity in meat sample obtained in the present study and predicted data calculated using Model 1 in Chapter 3. The result demonstrated a good agreement between experimental and predicted data which suggest that the model developed in Chapter 3 can satisfactorily predict residual cathepsin B and L activity in beef brisket during *sous vide* cooking. Even though the model in Chapter 3 was developed based on residual cathepsin B and L activity that was obtained by combining the meat residue and cook loss fraction, Figure 4.11 shows it can also satisfactorily predict residual cathepsin B and L activity in meat residue fraction. This is due to the fact that only small proportion of cathepsin B and L are expelled from the meat with the cooking loss during the heat treatments (Figure 4.9b). Residual cathepsin B and L activity in meat residue fraction is particularly important as it remains within the meat thus can contribute to the proteolysis of meat proteins during *sous vide* cooking.

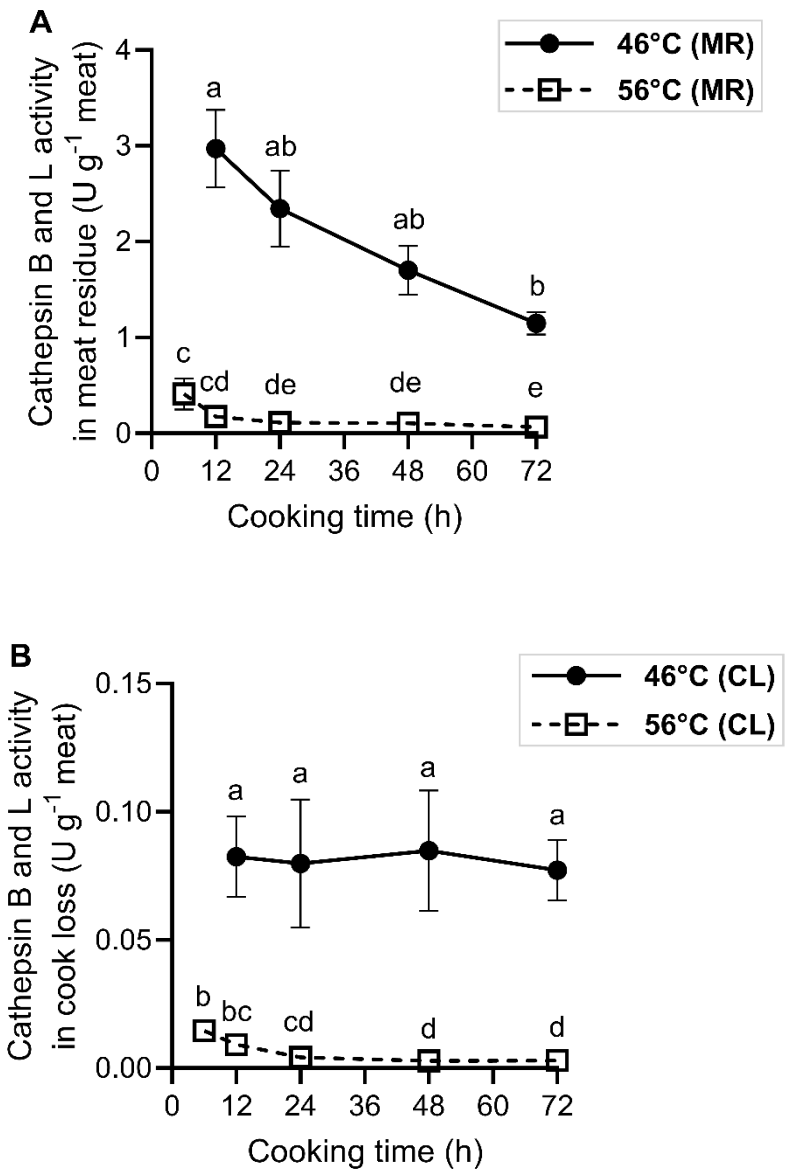

Figure 4. 9 Residual cathepsin B and L activity in **A.** meat residue (MR) **B.** cook loss (CL) fraction of beef brisket *sous vide* cooking at 46 and 56 °C at different cooking times. Symbols are means (n=3). Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). Different letters (a-e) statistically indicate the significant differences among treatments ($p < 0.05$).

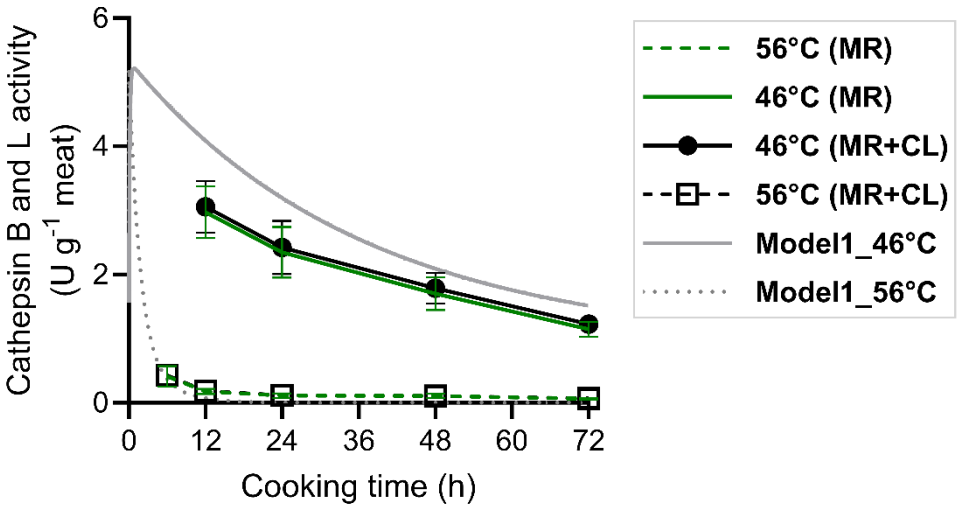

Figure 4. 10 Comparison between experimental data of residual cathepsin B and L activity in meat sample (combine meat residue and cook loss fraction, MR+CL) and meat residue (MR) with predicted data obtained using Model 1 in Chapter 3.

The previous chapter has shown that temperature also effect cathepsin B and L rate of reaction. Moreover, prolonged cooking time at temperatures where cathepsin B and L are still active can produce increasing cumulative cathepsin B and L activity. Due to this, in the present study the effect of cathepsin B and L on meat tenderness was evaluated using cumulative activity rather than residual activity. Figure 4.11 presents the cumulative cathepsin B and L activity at temperature 46 °C and 56 °C. The result shows that the amount of cumulative cathepsin B and L activity at temperature 46 °C was higher than at 56 °C at all time point. At 46 °C, the cumulative cathepsin B and L activity was 2600 units after 6 h cooking and this value gradually increase with increasing cooking time where it reaches 18700 units after 72 h cooking. On the other hand, at 56 °C for 6 h, the cumulative cathepsin B and L activity is only half of that at 46 °C for 6 h cooking and the value become relatively constant thereafter. Even though there might be a slight increase in the cumulative activity at 56 °C with increasing cooking time after 6 h cooking (since there was still a small concentration of cathepsin B and L that was still active, as can be seen from Figure 4.9), their contribution might be small. This indicates that, cooking at 46 °C for prolonged time provides a greater benefit in terms of getting higher and increasing cumulative cathepsin B

and L activity compared to at 56 °C. While increasing cooking time at 56 °C after 6 h does not give any additional benefit in terms of getting more cumulative proteolytic activity.

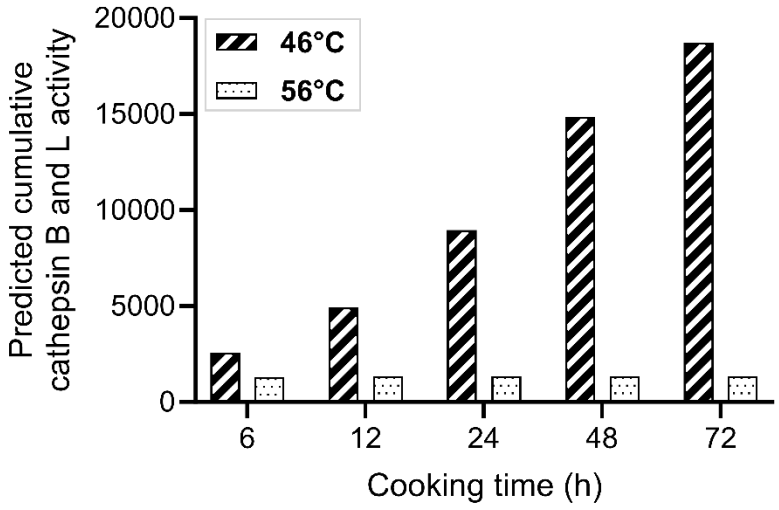

Figure 4. 11 Predicted cumulative cathepsin B and L activity (nmoles AMC g^{-1} meat) in beef brisket *sous vide* cooked at 46 °C and 56 °C.

4.3.3 Protein thermal denaturation

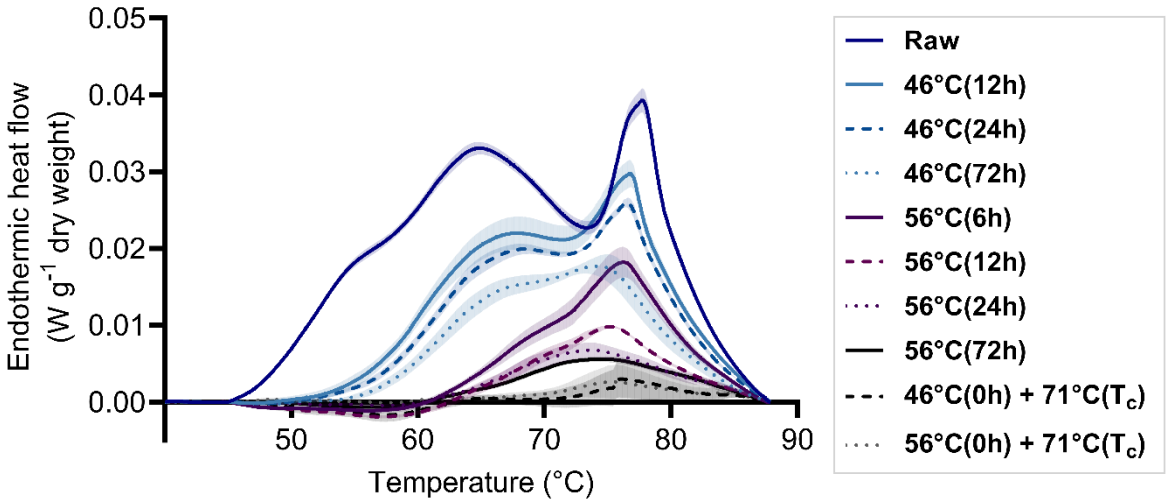

Figure 4. 12 DSC thermogram of raw and *sous vide* cooked beef brisket measured at heating rate of 5 °C min^{-1} after baseline subtraction. Lines are means (n=3). Shaded area above and below a line represent SEM. From top to bottom: Raw, 46°C(12h), 46°C(24h), 46°C(72h), 56°C(6h), 56°C(12h), 56 °C(24h), 5°C(72h), 46 °C(0h)+71°C, and 56 °C(0h)+71°C.

Figure 4.12 presents a typical DSC thermogram of meat sample, revealing three distinct endothermic peaks. At the DSC scanning rate of 5 °C min^{-1}, each peak has a T_{max} of 54.5 °C (Peak 1), 65.2 °C (Peak 2), and 77.5 °C (Peak 3) (Table 4.1). These peaks has been attributed to; Peak 1: light meromyosin (LMM) and heavy meromyosin subfragment-1 (HMM S-1), Peak 2: heavy meromyosin subfragment-2 (HMM S-2), sarcoplasmic proteins, and collagen, and Peak 3: actin (Bertazzon & Tsong, 1990; Liu, Puolanne, & Ertbjerg, 2014; Macfarlane, McKenzie, Turner, & Jones, 1981; Stabursvik & Martens, 1980; Wagner & Ańon, 1985) and titin (Pospiech, Greaser, Mikolajczak, Chiang, & Krzywdzińska, 2002). The result shows that heating treatments at both 46 °C and 56 °C result in the complete denaturation of proteins in Peak 1, as evidenced by the disappearance of Peak 1 from the DSC thermograms of cooked meat. Lower overall protein denaturation was observed at 46 °C compared to 56 °C. Cooking of meat to a centre temperature of 71 °C following cooking at 46 °C and 56 °C even for a shortest cooking time (0 h) produced complete protein denaturation.

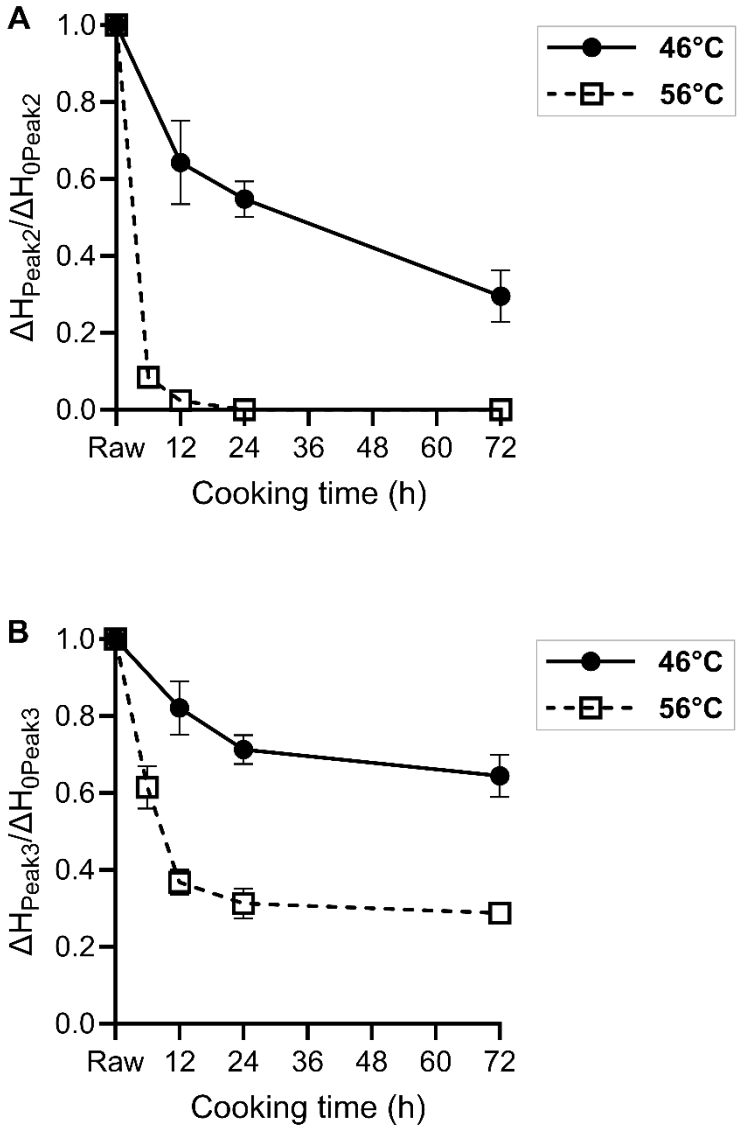

Figure 4. 13 Residual native proteins of **A.** Peak 2 and **B.** Peak 3 of beef brisket after *sous vide* cooked at 46 and 56 °C at different cooking times. Symbols are means (n=3). Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol).

Figure 4.13A and 4.13B shows the calculated residual native proteins of Peak 2 and Peak 3 after the heat treatments, respectively. At 46 °C, increasing cooking time cause a gradual decrease of Peak 2 native proteins where it reached about 70.4 % denaturation after 72 h of cooking. On the other hand, cooking at 56 °C cause a complete denaturation of Peak 2 proteins after 24 h cooking. Additionally, at 46 °C, only 35.6 % of the proteins Peak 3 denatured after 72 h cooking. While at 56 °C of Peak 3 protein shows around 71.2 % denaturation after 72 h cooking.

4.3.4 Peak shear force, shear force deformation curve, and cooking loss

In the present study, after meat was cooked at different heat treatments which represent different cumulative cathepsin B and L activity, meat sample was further cooked at 71 °C (Tc) before shear force measurement. The reason of meat sample was further cooked at 71 °C (Tc) was to allow any tenderisation obtained when cooking 46 and 56 °C to be compared under the similar degree of protein denaturation and water content (% cooking loss). As has been shown above, cooking at 46 °C and 56 °C results in different degrees of protein denaturation. Cooking at 46 °C exhibits minimal overall protein denaturation (Figure 4.12) and lower cooking loss compared to cooking at 56 °C (Figure 4.16). However, subsequent cooking of meat at 71 °C (Tc) following the cooking at 46 °C or 56 °C leads to complete protein denaturation, resulting in similar degrees of protein denaturation for the meat samples as can be seen in Figure 4.12 for 46 °C(0 h)+71 °C(Tc) and 56 °C(0 h)+71 °C(Tc) treatments. This is accompanied by an increase in the percentage of cooking loss for meat initially cooked at 46°C. At this cooking condition (subsequent cooking at 71 °C), there is no significant difference in cooking loss between meat cooked at 46 °C and 56 °C under similar cooking time (Figure 4.16). Tornberg (2005) proposed that the toughness of meat cooked at temperatures below 50 °C can be attributed to the viscous flow in the fluid-filled channels in between fibres and fibre bundles which reduced crack propagation and consequently tougher meat. On the other hand, at higher temperatures, gel formation becomes more favourable, effectively binding the fibres and fibre bundles together thus reducing the viscous flow. Thus, in order to minimize the influence of viscous dissipation when comparing the effects of cooking at 46 °C and 56 °C on meat tenderness, it is reasonable to apply subsequent cooking at 71 °C. Additionally, the suggested effects of cathepsin on preventing aggregation (Dominguez-Hernandez & Ertbjerg, 2021) and promoting the dissociation of actomyosin (Yao et al., 2023) on meat tenderness might be better observed when the meat was further cooked at higher temperatures such as 71 °C where aggregation and shrinkage are more prominent. Besides, cooking temperatures of 46 °C and 56 °C can be considered as low-temperature cooking. Practically, the meat will be seared or cooked further at a higher temperature before serving. In

this study, a higher cooking temperature of 71 °C (Tc) is chosen because it is a commonly used temperature for sensory measurement and objective tenderness evaluation of meat (AMSA, 2016).

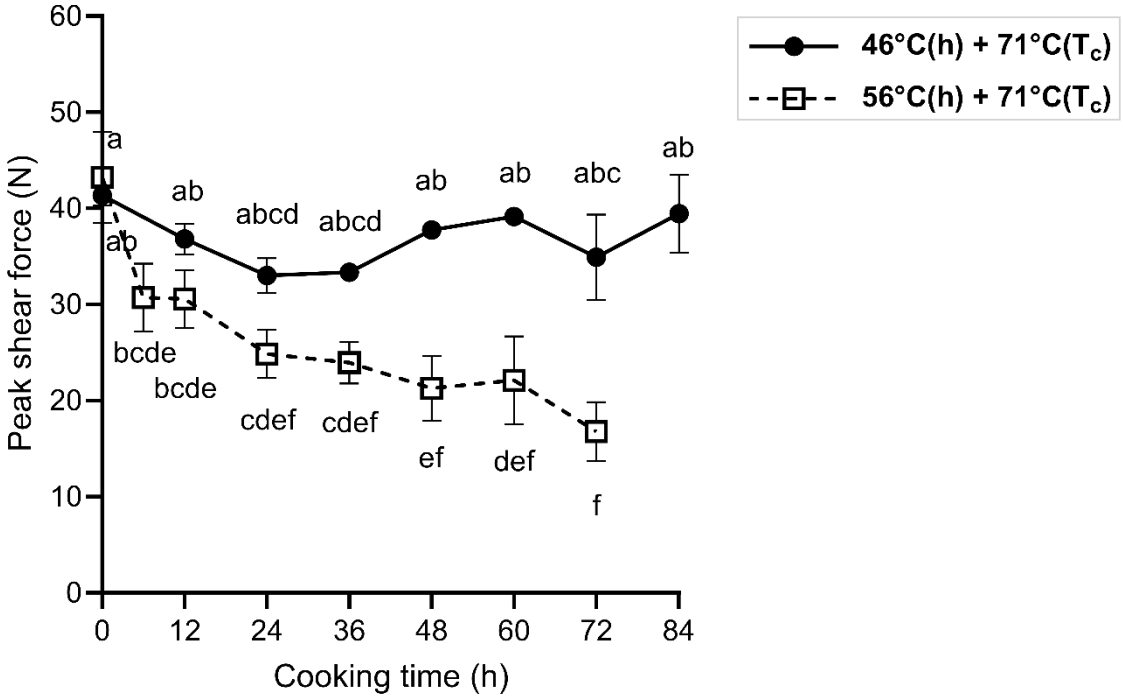

Figure 4. 14 Peak shear force (N) of beef brisket *sous vide* cooked at 46 and 56 °C at different cooking times and further *sous vide* cooked at 71 °C until core temperature (Tc) reaches 71 °C . Symbols are means (n=3). Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). Different letters (a-f) statistically indicate the significant differences among treatments ($p < 0.05$).

Figure 4.14 shows the peak shear force (N) for beef brisket *sous vide* cooked at 46 °C and 56 °C at different cooking time followed by further cooking at 71 °C (Tc). There was a significant effect of temperature x time interaction on peak shear force value ($p < 0.001$). The meat precooked at 46 °C and 56 °C for 0 h has a similar peak shear force value. Increasing cooking time at precooking temperature of 46 °C cause no significant change. On the other hand, for meat precooked at 56 °C, a significant decrease in peak shear force value was observed with increasing cooking time from 0 h (43 N) to 6h (31 N) which bring about 12 N (29 %) reduction from the

initial peak shear force value (0 h). Further increasing cooking time at this temperature cause a further decrease in the peak shear force value and significant after 72 h cooking (16 N) where it reached another 15 N (32 %) reduction from 6 h cooking. This brings about total 27 N (61 %) reduction from the initial peak shear force value after cooking for 72 h. This result indicates that precooking at 46 °C activity does not provide significant tenderisation whereas significant tenderisation was observed with precooking at 56 °C. Cooking at 46 °C was selected to maximise the cumulate cathepsin B and L activity, but this did not result in a reduction in peak shear force with cooking time. Cooking at 56 °C was selected as this had significantly less cumulative cathepsin B and L activity than cooking at 46 °C but was found to significantly reduce peak shear force. In fact, the rapid deactivation of cathepsin at 56 °C, means that cooking beyond 6 h at this temperature does not result in an increase in cumulative cathepsin B and L activity, yet a significant decrease in peak shear force value is observed when cooking beyond 6 h.

Figure 4.15 shows the shear force deformation curve of beef brisket *sous vide* cooked at 46 and 56 °C for different cooking time, followed by further cooking at 71 °C (Tc). The distance of 0.866 cm on the plot is approximately the point where the tip of the V-shaped blade comes into contact with the meat sample. While 1.866 cm is approximately the distance where the tip already completely cut through the meat sample (Figure 4.6). Meat precooked at 46 and 56 °C for 0 h have similar shape of shear force deformation curve. However, increasing cooking times produce different deformation curves between these temperatures. The increased cooking time from 0 to 72 h led to a gradual decrease in the force required to initiate fracture (initial yield). This decrease occurs more rapidly and to a greater extent at 56 °C compared to 46 °C. In addition, the shape of the shear deformation curve shows that the force of meat precooked at 46 °C (for all time treatments after 0 h) did not decrease rapidly after the initial yield. Instead, it can be observed that an increasing force is required to extend the crack and ultimately cause the final failure. In contrast, for meat precooked at 56 °C, an increase in cooking time leads to a decrease in the force required to extend the crack. Furthermore, for meat precooked at 56 °C for 72 h, the force decreases rapidly after the initial yield, and the curve exhibits the characteristic of a single failure

rather than progressive failure seen in other heat treatments. It can be seen that increasing the cooking time at 56 °C results in a gradual decrease in the final yield forces. On the other hand, the final yield forces remain relatively constant with increasing cooking time at 46 °C. Overall, the results indicate no changes in shear force, initial yield, final yield, or crack propagation with prolonged cooking time at 46 °C. However, at 56 °C, reductions in shear force, initial yield, final yield, and improved crack propagation are observed with prolonged cooking time.

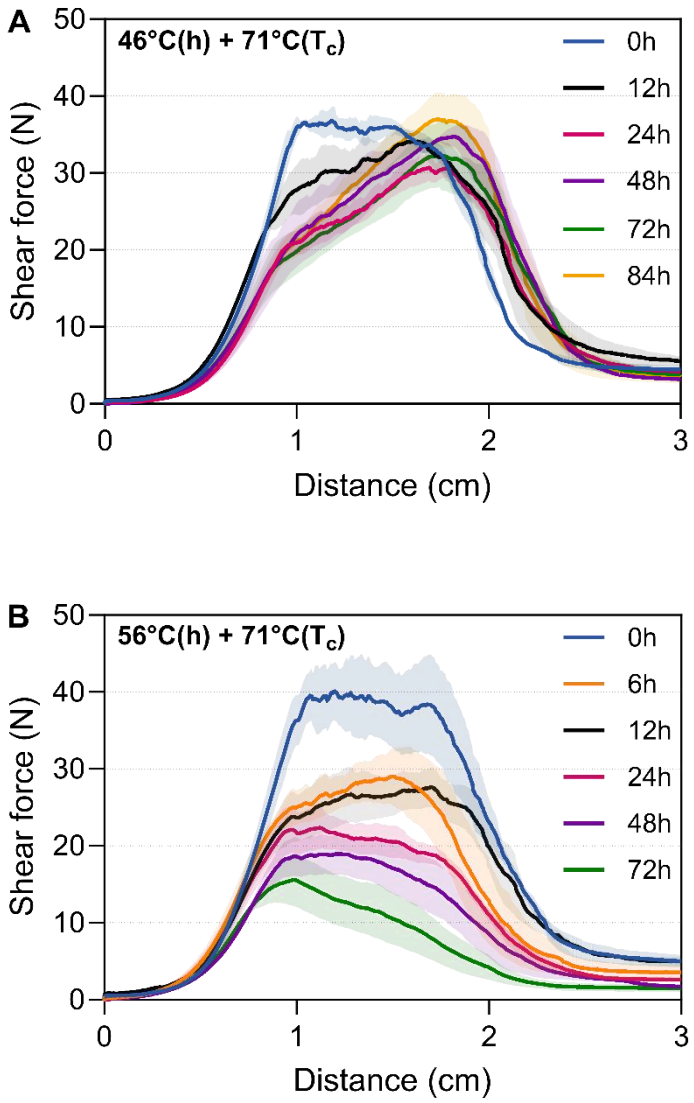

Figure 4. 15 Shear force deformation curve of beef brisket *sous vide* cooked at **A.** 46 °C and **B.** 56 °C at different cooking times and further *sous vide* cooked at 71 °C until core temperature (Tc) reaches 71 °C. Lines are means (n=3). Shaded area above and below a line represent SEM.

In addition to tenderness, juiciness is another important quality attribute that significantly influence consumer acceptance of cooked meat (Becker et al., 2016). As has been mentioned before, no significant difference was observed between precooking at 46 and 56 °C, with cooking loss range from 22.2 to 31.5 % (Figure 4.16). At both temperatures, increasing cooking time cause a gradual increase in cooking loss and significant after cooking for 84 h and 36 h at 46 °C and 56 °C, respectively.

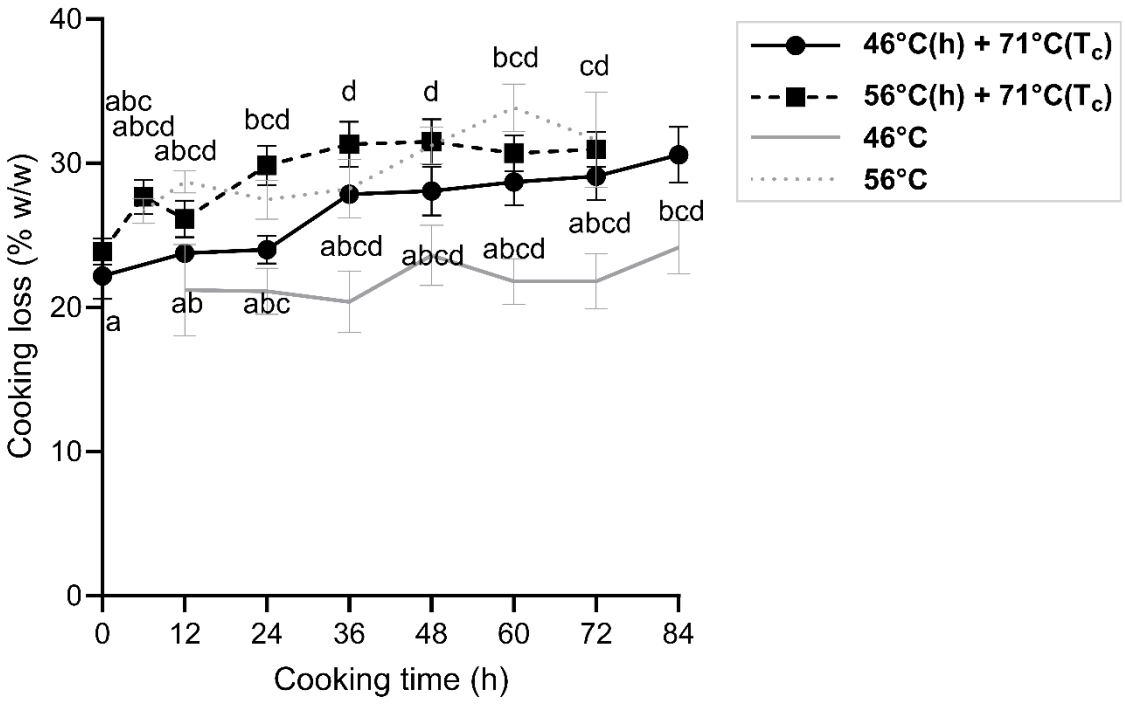

Figure 4. 16 Cooking loss (% w/w) of beef brisket *sous vide* cooked at 46 and 56 °C at different cooking times with and without further *sous vide* cooked at 71 °C until core temperature (Tc) reaches 71 °C . Symbols are means (n=3). Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). Different letters (a-d) statistically indicate the significant differences among samples further *sous vide* cooked at 71 °C(Tc) ($p < 0.05$).

4.3.5 Solubilised collagen in cook loss

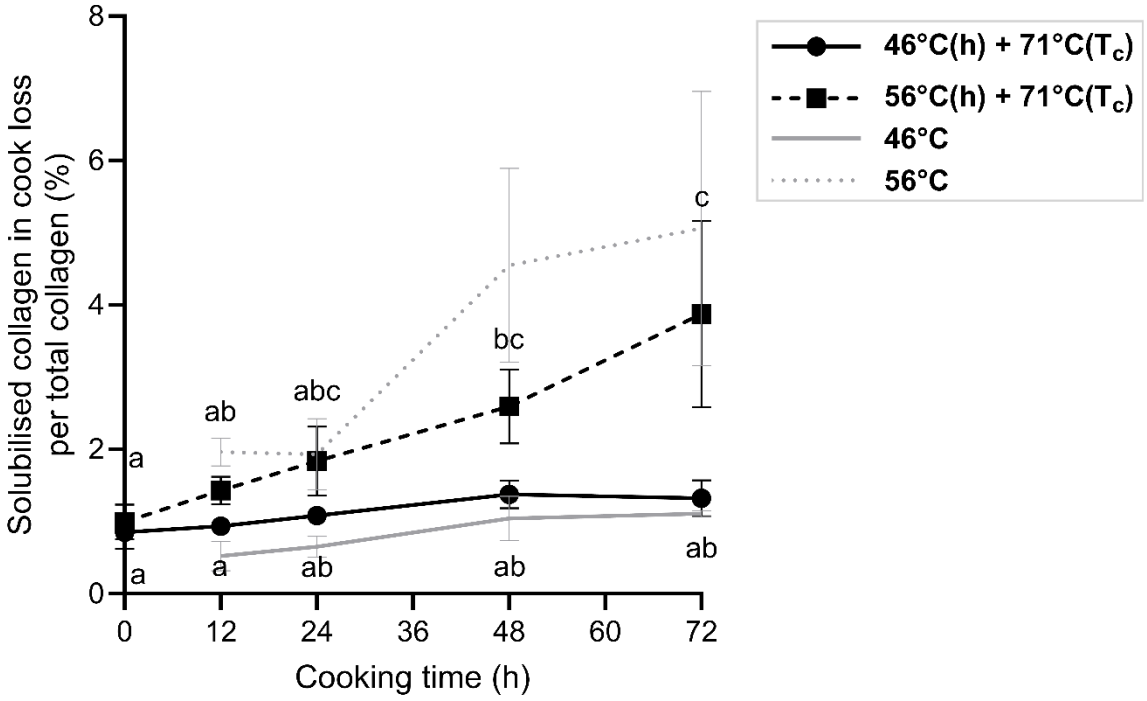

Figure 4. 17 Percentage of solubilised collagen in cook loss per total collagen (SC) of beef brisket *sous vide* cooked at 46 and 56 °C at different cooking times and with and without further *sous vide* cooked at 71 °C until core temperature (Tc) reaches 71 °C . Symbols are means (n=3). Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). Different letters (a-c) statistically indicate the significant differences among samples further *sous vide* at cooked 71 °C(Tc) ($p < 0.05$).

Figure 4.17 shows the percentage of solubilised collagen in cook loss per total collagen (SC) of beef brisket *sous vide* cooked at 46 °C and 56 °C for different cooking times with or without further cooking at 71°C (Tc). The result shows that the change in SC already occurs during the cooking at 46 and 56 °C rather than during the subsequent heat treatment at 71 °C. Additionally, there was a significant effect of temperature x time on SC ($p < 0.001$) for meat precooked at 46 and 56 °C. For meat precooked at 46 °C, there was no significant increase in SC with increasing cooking time. On the other hand, for meat precooked at 56 °C, a significant increase in SC was observed with increasing cooking time from 0 h (0.99 ± 0.24 %) to 48 h (2.6 ± 0.51 %). The value

of solubilised collagen in cook loss obtained in this study which are 0.8 to 3.9 % of solubilised collagen in cook loss per total collagen (Figure 4.17) and 0.1 to 0.4 mg solubilised collagen in cook loss per gram meat is in the range of value obtained for yak *longissimus lumborum* and beef *semitendinosus* muscle cooked at temperature between 50 to 63 °C for prolonged time (Christensen et al., 2013; Wang et al., 2022).

4.3.6 Correlation

Table 4. 3 Pearson correlation coefficients (r) between selected parameters of *sous vide* cooked beef brisket, N=24.

Parameter	Peak shear force	Predicted cumulative cathepsin B and L activity
Predicted cumulative cathepsin B and L activity	0.61**	-
Solubilised collagen in cook loss per total collagen (SC)	-0.82***	-0.37 (NS)
Cooking loss	-0.57**	-0.13 (NS)

NS = non-significant
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Correlations were carried out to determine the relationships between peak shear force and cumulative cathepsin B and L activity and other parameters of *sous vide* cooked beef briskets (Table 4.3). The scatter plot of the correlation was illustrated in Figure 4.18. Interestingly, the result shows that peak shear force value has a significant positive correlation with cumulative cathepsin B and L activity which indicates that precooking at higher cumulative cathepsin B and L activity is associated with tougher meat. On the other hand, a significant negative correlation was observed for peak force value with SC and cooking loss, while no significant correlation was observed for predicted cumulative cathepsin B and L activity with SC and cooking loss.

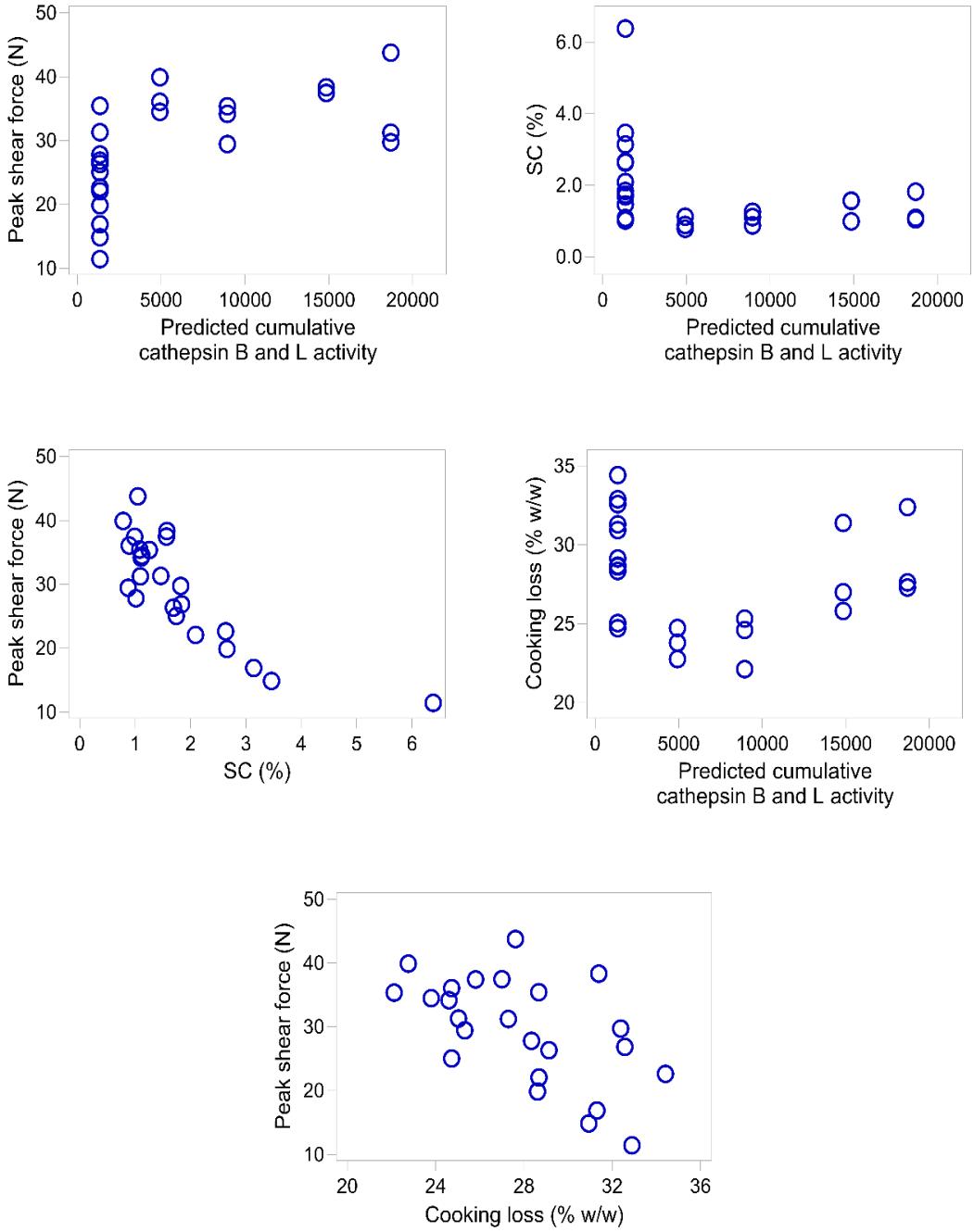

Figure 4. 18 Scatter plots of selected parameters of *sous vide* cooked beef brisket, N=24.

Abbreviations: solubilised collagen in cook loss per total collagen (SC).

4.4 Discussion

The result in this study is in agreement with the previous chapter that shows that cooking at 46 °C produce slower rate of cathepsin B and L deactivation thus higher cumulative cathepsin B and L activity over time compared to cooking at 56 °C. Besides, the analysis of meat proteins thermal denaturation using DSC shows that LMM and HMM S-1 myosin fragments already denatured at 46 °C cooking which can produce greater potential of myosin proteolysis by cathepsin B and L (Schwartz and Bird (1977). Thus, it is expected that precooking at 46 °C can contribute to meat tenderisation. However, the result shows that precooking at 46 °C did not result in significant decrease in peak force value. On the other hand, significant decrease in peak force value was obtained in meat precooked at 56 °C which represent low cumulative cathepsin B and L activity. Further correlation analysis also shows a significant positive correlation between cumulative cathepsin B and L activity and peak shear force value (0.61; $p<0.01$) (Table 4.3).

The reason behind the lack of tenderisation of meat precooked at 46 °C is unclear and requires further investigation. One of the reasons could be due to limited or absence of myofibrillar proteolysis during *sous vide* cooking. Although it is possible that complete myofibrillar proteolysis has already occurred during post-mortem aging, potentially limiting any further myofibrillar proteolysis during *sous vide* cooking, previous studies have reported that within 3 – 7 days of post-mortem aging, only large myofibrillar proteins such as titin and nebulin are completely degraded (Huff-Lonergan et al., 1996; Lomiwes, Farouk, et al., 2014; Wu, Clerens, & Farouk, 2014). On the other hand, the degradation of myosin heavy chain, actin, troponin-T, and desmin has been shown to require a longer time to complete. For example, intact troponin-T and desmin were still observed at 14 to 28 days post-mortem (Dang et al., 2020; Huff-Lonergan et al., 1996; Lomiwes, Farouk, et al., 2014). Similarly, Gagaoua, Troy, and Mullen (2021) reported that the intensity 110 kDa and 30 kDa protein fragments, which are degradation products of myosin heavy chains, and troponin-T and actin, respectively, still increase with increasing aging time from 7 to 14 days. In the present study, 4-days aged beef brisket was used, suggesting that there is still an opportunity for cathepsin B and L to act upon these myofibrillar proteins and

contribute to further tenderisation during *sous vide* cooking. Furthermore, tenderness measurement of meat cooked at 71 °C(Tc) showed that the beef brisket used in this study is considered as tough meat cut and has high shear force value (45.1 N) (Table 4.1), indicating the potential for improved tenderness through the cooking process. However, since no tenderisation was observed, it could be that myofibrillar proteolysis might not occur during *sous vide* cooking or only occur to a limited extent thus not contributing to a significant tenderisation. This view is supported by the result obtained by Li et al. (2019) that shows that the appearance of 30 kDa protein fragment, an important marker of myofibrillar proteolysis, is significantly influenced by aging but not heating treatments. They reported that regardless of heat treatments (53 and 58 °C for 10 and 20 h), 10-day aged pork loin cooked has significantly higher amount of 30 kDa fragments compared to 1-days aged pork loin, indicating that heat treatments do not promote myofibrillar proteolysis. Similarly, Ertbjerg et al. (2012) demonstrated that meat cooked at 55 °C for 24 h that represent high cathepsin B and L residual activity only produce minor desmin degradation.

The result of shear force deformation curve also supports the above arguments. If myofibrillar proteolysis by cathepsin B and L occurs during *sous vide* cooking, greater and more rapid decrease in initial yield value would be expected in meat precooked at 46 °C compared to 56 °C. Bouton et al. (1975) suggested that initial yield forces mainly reflected the myofibrillar strength. In the present study there was no greater and faster myofibrillar tenderisation observed with precooking at higher cumulative cathepsin activity since the decrease in the initial yield force is greater and more rapid at lower cumulative cathepsin activity compared to higher cumulative activity (Figure 4.15).

Another reason for the lack of tenderisation in meat precooked at 46 °C could be that myofibrillar proteolysis occurs but is masked by the toughness of connective tissue, as supported by the relatively constant final yield value (associated with connective tissue strength) of meat precooked at 46 °C (Moller, Sorensen, & Larsen, 1981). This could be attributed to low collagen solubilisation at this heating treatments (Figure 4.17). Moreover, there is a possibility that cooking

at 46 °C only produce minimal collagen denaturation thus limits the connective tissue proteolysis by cathepsin B and L. Etherington (1974) reported that denatured collagen is more susceptible to proteolytic digestion compared to native collagen. Even though the protein thermal denaturation analysis shows that cooking at 46 °C for 72 h causes a 70 % reduction in Peak 2 enthalpy of denaturation, Peak 2 also consists of several other proteins beside collagen. As a result, the degree of collagen denaturation cannot be determined directly from Peak 2 thermal denaturation data, thus it was investigated in the next study.

Comparison between the degree of collagen denaturation obtained from Peak 2 of the DSC thermogram (Figure 4.12) and the degree of collagen solubilisation (Figure 4.17) shows that complete disappearance of Peak 2 was observed after 24 h of cooking at 56 °C. However, gradual increase in the SC was still observed after 24 h and significant in meat precooked at 56 °C for 48 h. The difference in time course between collagen denaturation and solubilisation support the view that collagen solubilisation has a different kinetic process than collagen denaturation (Bernal & Stanley, 1987). Collagen denaturation involves the disruption of hydrogen bonds within the collagen triple helix, resulting in the conversion of collagen triple-helix into a random coil structure. However, for meat with high concentration of heat-stable cross-links, the denatured collagens remained connected by cross-links thus insoluble and retained within the meat unless more intense cooking treatment is applied (Christensen et al., 2013; Mohr & Bendall, 1969). Several studies have shown that higher cooking temperatures and longer cooking times cause a decrease in the proportion of γ- (three cross-linked α-chains) component of collagen (Pan et al., 2018; Wang et al., 2022). This reduction is accompanied by an increase in the proportion of β- (two cross-linked α-chains), α-chains and lower molecular weight peptides, indicating an enhanced breakdown of inter and intramolecular collagen cross-links, and peptide bonds under more intense cooking conditions. Wang et al. (2022) reported that increasing cooking temperature of yak longissimus muscle from 50 °C to 60 °C for 4 h led to a decrease in the γ-component, accompanied by an increase in the β-components and smaller molecular weight peptides of 75 and 35 kDa. Similarly, Pan et al. (2018) reported that increasing heating treatment of fish skin

from 45 °C to 55 °C for 6 and 12h cause the decrease in γ-component and β-components, along with an increase in lower molecular weight peptides of 100–50 kDa. This was also linked with an increase in collagen extraction yield (Pan et al., 2018). Therefore, it is suggested that the increase in SC with extended cooking time at 56 °C is also indication of a greater hydrolysis of collagen cross-link/ collagen, resulting in the formation of lower molecular weight collagen monomers/ peptides that are readily solubilised, thus increasing the possibility to migrate out of the meat along with water.

Furthermore, in this study, a significantly high negative correlation (–0.82; $p<0.001$) was obtained between peak shear force value and collagen solubilisation (Table 4.3). The increase in collagen solubilisation was also accompanied by a gradual decrease in the final yield force, which corresponds to the connective tissue strength (Møller, 1981). These results are in agreement with previous observation that demonstrates that collagen solubilisation contribute to meat tenderness during *sous vide* cooking (Christensen et al., 2011; Christensen et al., 2013; Naqvi et al., 2021). In addition, in this study we also showed that increasing precooking time from 0 to 72 h at 56 °C cause 61 % (27 N) reduction in the shear force value. However, at this cooking treatments only 2 – 4 % of total collagen exude from the meat with cooking loss, which mean 96 – 98 % of collagen still retain within the meat. Given the significantly high total collagen content found in beef brisket, which has been demonstrated to be three times greater than that of more tender muscles such as psoas major (Torrescano et al., 2003), the relatively small reduction in collagen quantity resulting from collagen solubilisation may not be the only factor contributing to the observed increase in tenderness. Similarly, Fareh (2018) also pointed out that the limited amount of solubilised collagen (10 %) could not justify the observed tenderness of beef *semitendinosus* muscle cooked at 60 °C to 90 °C for extended time. As has been proposed above, the increase in collagen solubilisation might also indicate greater collagen hydrolysis. Thus, collagen inside the meat might compose of relatively higher proportion of low-molecular-weight peptide. Several studies have reported that the mechanical property of gelatin is influenced by the molecular weight of collagen peptides (Pan et al., 2018; Tang et al., 2023). Pan et al. (2018) reported that

gelatin produce from low molecular weight peptide has a lower gel strength compared to gelatin produce from high molecular weight peptides. Thus, it is proposed that the decrease in the peak shear force value with increasing precooking time at 56 °C might also be explained by the increase in the proportion of low-molecular-weight collagen peptides inside the meat. These peptides form a weak gelatin structure, resulted in improved meat tenderness. A similar mechanism has been suggested by Tang et al. (2022) to explain the increase in tenderness of chicken claw cooked at 90 to 121 °C for 10 to 30 min. They demonstrated that the decrease in the hardness of the chicken claw with increasing cooking treatment is linked to the increase in collagen solubilisation and the proportion of low-molecular-weight collagen peptides.

4.5 Conclusion

Precooking at 56 °C resulted in significant meat tenderisation, while precooking at 46 °C did not. The increased tenderisation in meat precooked at 56 °C was associated with a higher level of collagen solubilisation compared to at 46 °C. The lack of tenderisation in meat precooked at 46 °C suggests that greater cumulative cathepsin B and L activity does not support tenderisation. This could be due to the absence of myofibrillar proteolysis even in the presence of cathepsin B and L.

Another possible explanation is the tenderisation due to myofibrillar proteolysis is masked by connective tissue toughness due to the low collagen solubilisation in meat precooked 46 °C. Additionally, minimal collagen denaturation at the 46 °C treatment might also affect connective tissue proteolysis, as the triple helix structure of native collagen might prevent the accessibility of cathepsin B and L to the cleaving site, thus contributing to connective tissue toughness. Even though the DSC profile shows that cooking at 46 °C produces 70 % denaturation of proteins in Peak 2, it is important to note that Peak 2 also consists of several other proteins besides collagen. As a result, the degree of collagen denaturation cannot be determined directly from the Peak 2 thermal denaturation data thus was further evaluated in Chapter 5 together with the kinetics of collagen thermal denaturation. Subsequently, the effects of cooking at varying levels of

cumulative cathepsin B and L activity and degree of collagen denaturation followed by cooking at temperature treatments that can promote collagen solubilisation on meat tenderness was investigated in Chapter 6.

CHAPTER 5 Modelling of collagen thermal denaturation in beef brisket

5.1 Introduction

Variation in meat tenderness among muscles is associated with their total collagen content. Despite contradictory findings that show both high and low correlations between meat tenderness and collagen content, especially when the correlation is conducted across different muscle cuts (Jeremiah et al., 2003; Rhee, Wheeler, Shackelford, & Koohmaraie, 2004), Hammond et al. (2022) reported that each muscle cut has a specific tenderness factor. In the case of beef brisket, tenderness is significantly related to collagen content.

During cooking, thermal denaturation of collagen has been associated with the change in meat texture and cooking loss. Bouton and Harris (1972) suggested that the tenderisation of meat, with increasing cooking temperature from 50 to 60 °C, could be attributed to collagen denaturation. Furthermore, Tornberg (2005) suggested that the cooperative shrinkage of collagen and actin contributes to cooking loss, especially at temperatures between 60 and 70 °C. Due to these contradictory findings, a study to determine the effect of temperature and time on collagen denaturation, especially under prolonged cooking times, which can be used to relate to the changes in texture and cooking loss of meat during cooking, is warranted.

Furthermore, numerous studies have reported that the incubation of cathepsin B and L with native collagen cause an increase in the amount of solubilised collagen (Burleigh et al., 1974; Kirschke et al., 1982; Maciewicz, Etherington, Kos, & Turk, 1987). These enzymes degrade collagen at the telopeptide (non-helical) region of the collagen molecule. Since the collagen cross-links are located in the telopeptide regions, the degradation of collagen in this region eliminates collagen cross-links, leading to collagen solubilisation (Burleigh et al., 1974; Kirschke et al., 1982). Additionally, it has been reported that the rate of collagen degradation by cathepsin is higher in denatured collagen compared to native collagen. This is suggested due to the lack of triple helix conformation in denatured collagen which increase the susceptibility of collagen telopeptide regions to proteolytic attack (Shoji et al., 2014).

Despite several studies has been done to characterise collagen denaturation in cooked meat, no kinetic models have been developed to predict collagen thermal denaturation, especially in intact meat (Christensen et al., 2013; Stabursvik & Martens, 1980; Zielbauer et al., 2016). Bertola, Bevilacqua, and Zaritzky (1994) determined the kinetics of collagen thermal denaturation using the data of peak shear force value associated with the tenderisation phase. Kajitani, Fukuoka, and Sakai (2011) reported the kinetics of thermal denaturation of meat proteins in cured pork sirloin. Latorre et al. (2018a) reported the kinetics of collagen thermal denaturation in isolated perimysium of beef *semitendinosus* and *pectoralis profundus* muscle. However, it is generally known that the thermal denaturation of proteins is affected by environmental factors such as pH, ionic strength, and the present of other cellular components, thus the kinetics of collagen thermal denaturation in cured meat and isolated perimysium might be different from those in intact meat. Therefore, the objective of this study is to determine the kinetics of collagen thermal denaturation in intact beef brisket. The kinetics data obtained in this study can be used to understand the effect of thermal denaturation of collagen on meat quality such as tenderness and cooking loss especially under prolonged cooking time such as during *sous vide* cooking. Besides, the data can be used in combination with the kinetics of cathepsin B and L activity to design *sous vide* processing conditions that can promote collagen proteolysis and collagen solubilisation.

5.2 Materials and methods

5.2.1 Sample preparation

Two beef briskets were obtained from a local butcher (Mad Butcher) and a meat processing plan (Taylor Preston Limited) in two separate dates, one in March 2022 (Brisket 2) and another one in June 2022 (Brisket 3). The external fat and connective tissue were first trimmed off from each brisket that was then cut across the muscle fibre into slices of 2 mm in thickness. A thin size of the meat samples was selected to allow rapid heating and a near isothermal condition during *sous vide* cooking. The slices were then individually vacuum packed and stored overnight at 4 °C before being processed on the next day.

The samples were *sous vide* treated at the temperatures of 50 °C, 53 °C, and 56 °C for times ranging from 0.5 – 72 h, making a total of 15 temperature-time combinations. Cooking was stopped by immersing samples into ice water baths. The sample was stored in -20 °C until further analysis (Ngapo et al., 1999).

For Brisket 1, the result was obtained from Chapter 4. The sample and sample preparation were described in Sections 4.2.1, 4.2.2, 4.2.3, and 4.2.4. This gives another 9 temperature-time combinations. Combining these with Brisket 2 and Brisket 3 produces a total of 26 temperature-time combinations.

5.2.2 pH measurement

pH measurement was carried out as described in Chapter 3, section 3.2.3.

5.2.3 Differential scanning calorimetry (DSC) analysis

DSC analysis was carried out as described in Chapter 4, section 4.2.10.

5.2.4 Kinetic models

In this study, the kinetics of collagen thermal denaturation was determine using the data of the change in the residual enthalpy of denaturation of Peak 2 (ΔH/ΔH0). Miles and Bailey (1999) proposed that the kinetics of collagen denaturation is governed by an irreversible rate process in which native collagen is transformed into a denatured state via a highly temperature-dependent rate constant. This process can be described using a first-order reaction model.

$$\boldsymbol{N \overset{k_1}{\rightarrow} D} \tag{5.1}$$

Where, N = native protein, D = denatured protein, k_1 = rate of denaturation.

$$\boldsymbol{\frac{d[N]}{dt} = -k_1[N]} \tag{5.2}$$

Integrated rate equation, at t=0, $[N]=[N]_0$

$$\boldsymbol{\frac{[N]}{[N]_0} = e^{-k_1 t}} \tag{5.3}$$

However, since the Peak 2 of meat DSC thermograms not only comprises collagen but also other meat proteins which are HMM S-2 and sarcoplasmic proteins, the kinetics of collagen thermal denaturation was determined using three-protein model. In this model, Peak 2 consists of three proteins which are N_1, N_2, and N_3 which represents HMM S-2, sarcoplasmic proteins, and collagen, respectively, where $N_1 + N_2 + N_3 = 1$, each has different thermal stability, and denatures according to a first-order reaction (Stabursvik & Martens, 1980). The kinetics of thermal denaturation of three-protein model can be describe as Equation (5.4).

. $$\boldsymbol{[N]\begin{cases}[N_1] \overset{k_{N1}}{\longrightarrow} [D_1] \\ [N_2] \overset{k_{N2}}{\longrightarrow} [D_2] \\ [N_3] \overset{k_{N3}}{\longrightarrow} [D_3]\end{cases}} \tag{5.4}$$

Where, N = native proteins in Peak 2, N_1 = native protein 1 (HMM S-2), N_2 = native protein 2 (sarcoplasmic proteins), N_3 = native protein 3 (collagen), D_1 = denatured protein 1, D_2 = denatured

protein 2, D_3 = denatured protein 3, k_{N1} = rate of denaturation of protein 1, k_{N2} = rate of denaturation of protein 2, k_{N3} = rate denaturation of protein 3.

$$\frac{d[N]}{dt} = -k_{N_1}[N_1] - k_{N_2}[N_2] - k_{N_3}[1 - N_1 - N_2] \quad \textbf{(5.5)}$$

$$\frac{d[D_1]}{dt} = -k_{N_1}[N_1]$$

$$\frac{d[D_2]}{dt} = -k_{N_2}[N_2]$$

$$\frac{d[D_3]}{dt} = -k_{N_3}[N_3]$$

Integrated rate equation, at t = 0, [N] = $[N]_0$, $[D_1]_0 = [D_2]_0 = [D_3]_0 = 0$ (Aymard & Belarbi, 2000):

$$\frac{[N]}{[N]_0} = N_1 e^{-k_{N1}t} + N_2 e^{-k_{N2}t} + (1 - N_1 - N_2) e^{-k_{N3}t} \quad \textbf{(5.6)}$$

$$[D_1] = [N_1]_0 \left(1 - exp^{-k_{N1}t}\right)$$

$$[D_2] = [N_2]_0 \left(1 - exp^{-k_{N2}t}\right)$$

$$[D_3] = [N_3]_0 \left(1 - exp^{-k_{N3}t}\right)$$

5.2.5 Data fitting and goodness of fit

Data fitting and goodness of fit was carried out as described in Chapter 3, section 3.2.10. In this study, experimental data from this study was combined with DSC data from Chapter 4 (assigned as Brisket 1), making a total of 22 temperature-time combinations. The data of the residual native protein of Peak 2 ($\Delta H/\Delta H_0$) were fit simultaneously to Equation (5.3) and Equation (5.6) using reparameterization of the Arrhenius equation. The temperature of 52 °C was chosen as reference (T_{ref}).

The value of parameters N_1 (HMM S-2), N_2 (sarcoplasmic proteins), and N_3 (collagen) from Equation 5.6 were fixed based on data of meat composition obtained from the literatures. Meat composes of 5.5 % myosin, 5.5 % sarcoplasmic proteins, and 1 % collagen on a wet weight basis (López-Bote, 2017). HMM S-2 is a sub-fragment of myosin molecule, both have molecular weight of 100 kDa (Liu, Puolanne, et al., 2014) and 520 kDa (Guo & Greaser, 2022), respectively.

Additionally, sarcoplasmic proteins contain thermal labile enzymes such as phosphorylase b, creatine kinase, triose phosphate isomerase, and myokinase. These enzymes have been shown to be denatured at low temperature 37 – 40 °C, thus are not included in Peak 2 of DSC thermogram (Joo, Kauffman, Kim, & Park, 1999; Liu, Ruusunen, Puolanne, & Ertbjerg, 2014; Scopes, 1964). Phosphorylase b, creatine kinase, and triose phosphate isomerase and myokinase make up 13.2, 7.6, and 4 % of sarcoplasmic proteins respectively (Marino et al., 2014). Based on the above information, the concentration of HMM S-2, sarcoplasmic proteins (excluding the heat labile enzymes), and collagen was calculated based on ratio where HMM S-2 + sarcoplasmic proteins + collagen = 1, the value obtained were 0.1707, 0.6679, and 0.1614, respectively. Thus, for three-protein model, only 6 parameters (E_{aN1}, E_{aN2}, E_{aN3}, $k_{52°CN1}$, $k_{52°CN2}$, and $k_{52°CN3}$) were fitted while 2 parameters were fixed (N_1 and N_2,).

5.2.6 Simulation of the change in denatured proteins

The changes in the fraction of the denatured proteins present in Peak 2 were simulated at temperature 46, 50, 53, 56, 60, and 70 °C according to kinetic parameters obtained from the three-protein model.

5.3 Results and discussion

5.3.1 Characteristic of raw beef brisket

Table 5. 1 Characteristic of raw beef brisket. Values are mean ± standard error of mean (SEM).

Meat properties	Brisket 1 n=3	Brisket 2 n=1	Brisket 3 n=1
Breed	Hereford-Friesian cross	–	Angus/ Hereford
Sex	Heifer	–	Heifer
pH	5.48 ± 0.02	5.50	5.50
Post-mortem day	4 days	14 days	18 days

Table 5.1 shows the characteristics of beef briskets used in this study. All the three briskets have a similar pH value which is in the range of normal post-rigor pH value of beef. Brisket 1 and 3 were Hereford-Friesian cross and British heifers (Angus/ Hereford), respectively. There was no available information on the breed and sex of Brisket 2. Brisket 1, 2, and 3 were *sous vide* cooked at 4 days, 14 days, and 18 days post-mortem, respectively.

Figure 5.1A shows that the three briskets produced a similar shape for the DSC thermograms with three main peaks. Peak 1 has been assigned to light meromyosin (LMM) and heavy meromyosin subfragment-1 (HMM S-1), Peak 2 to heavy meromyosin subfragment-2 (HMM S-2), sarcoplasmic proteins, and collagen, and Peak 3 to actin (Bertazzon & Tsong, 1990; Liu, Puolanne, et al., 2014; Macfarlane et al., 1981; Stabursvik & Martens, 1980; Wagner & Añon, 1985) and titin (Pospiech et al., 2002). Brisket 2 and 3 show a slightly higher peak temperature (T_{max}) and denaturation enthalpy (ΔH) for Peak 2 compared to Brisket 1 (Table 5.2) which could be due to variation in the meat samples used. Overall, the mean of Peak 2 T_{max} and ΔH of the three briskets were 66.8 °C and 6.07 J g^{-1} dry weight, respectively.

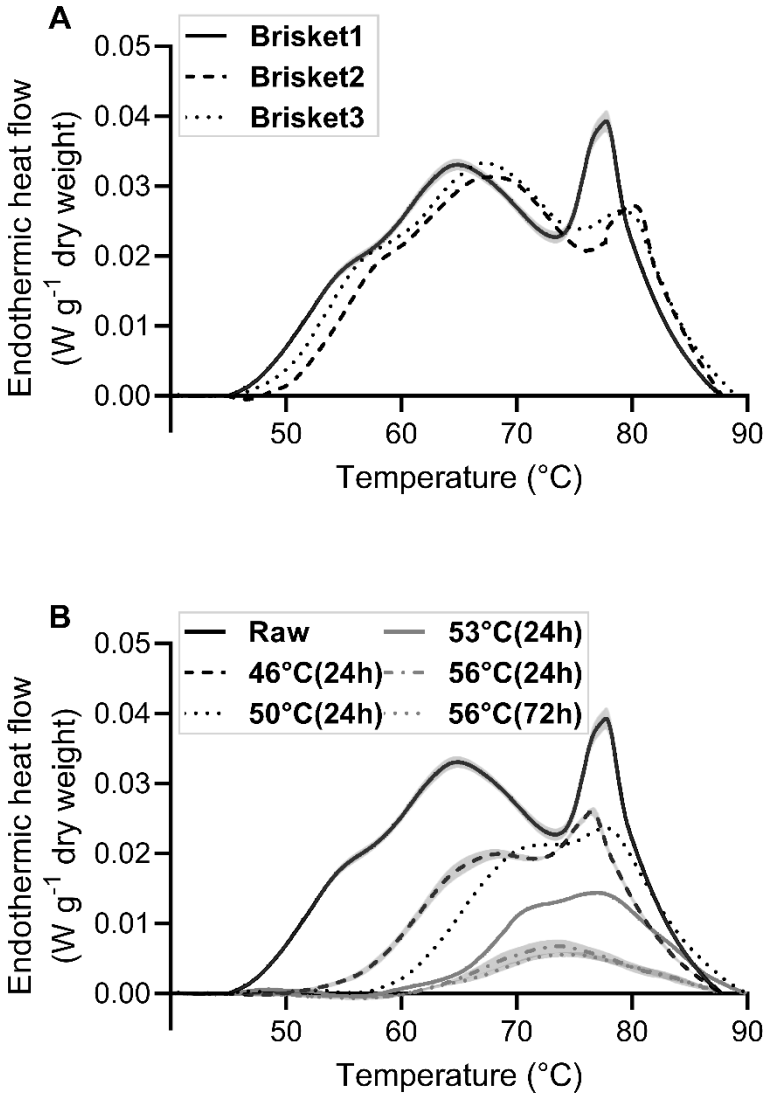

Figure 5. 1 A. DSC thermogram of raw beef briskets measured at heating rate of 5 °C min-1 after baseline subtraction. For Brisket 1, lines are means (n=3) and shaded area above and below the line represent SEM. Brisket 2 and Brisket 3 have only one replicate. **B.** A representative of DSC thermogram of raw and *sous vide* cooked beef brisket measured at heating rate of 5 °C min-1 after baseline subtraction. From top to bottom: Raw, 46 °C(24h), 50 °C(24h), 53 °C(24h), 56 °C(24h), and 56 °C(72h). Lines are means (n=3) and shaded area above and below the line represent SEM. Lines that do not have shaded area means only one replicate was used.

The area under DSC thermograms represent the total energy involved in the protein denaturation or known as enthalpy of denaturation (ΔH) (Bischof & He, 2006). The application of heat treatment causes meat proteins to denature and consequently a reduction in the concentration of remaining native proteins. This can be seen as the reduction of area under the curve of DSC thermogram of cooked meat compared to raw meat (Figure 5.1B). Thus, the data of the enthalpy of denaturation (ΔH) of specific peak after heat treatment compared to that of raw meat (ΔH_0) can be used to determine the kinetics of thermal denaturation of proteins associated with that peak (Bertola et al., 1994; Martens et al., 1982; Miles, Burjanadze, & Bailey, 1995; Parsons & Patterson, 1986; Verhaeghe et al., 2016). Thus, in this study, the data of the change in the enthalpy of denaturation of Peak 2 ($\Delta H/\Delta H_0$) was used to determine the kinetics of collagen thermal denaturation.

Table 5. 2 Peak temperature (T_{max}) and denaturation enthalpy (ΔH) of raw beef brisket obtained from DSC thermogram at heating rate of 5 °C min^{-1}. Values are mean ± SEM. Brisket 2 and Brisket 3 have only one replicate.

Sample	T_{max} (°C)			Enthalpy(J g^{-1} dry weight)		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
Brisket 1	54.5 ± 0.3	65.2 ± 0.1	77.5 ± 0.1	1.47 ± 0.20	5.13 ± 0.40	3.50 ± 0.13
Brisket 2	57.3	67.9	80.1	0.77	6.58	1.58
Brisket 3	56.2	67.4	79.8	1.20	6.50	2.06
Mean	56.0 ± 0.8	66.8 ± 0.8	79.1 ± 0.8	1.15 ± 0.20	6.07 ± 0.47	2.38 ± 0.58

5.3.2 Kinetics of thermal denaturation for Peak 2

Figure 5.2 and 5.3 present the residual enthalpy of denaturation of Peak 2 in beef brisket *sous vide* cooked at 46 to 56 °C for different cooking time, fitted to the single first-order reaction and three-protein model, respectively. The result of non-linear regression using a first-order model (Figure 5.2) shows that the model explained lower variation of the thermal denaturation data compared with the three-protein model, which was supported by the lower R^2 = 0.836 (Table 5.3). The

activation energy was 392 kJ mol^{-1} and rate constant at 52 °C was $k_{52°C}$ = 0.0054 min^{-1}. On the other hand, the result of nonlinear regression using the three-protein model shows a better fit of the data in Figure 5.3 and a higher R^2 = 0.926 compared to the single first-order reaction model. This model assumed that enthalpy denaturation of Peak 2 consists of thermal denaturation of three proteins which are N_1, N_2, and N_3 which have been assigned to HMM S-2, sarcoplasmic proteins, and collagen, where each has different thermal stability and denatures according to a first-order reaction. As has been discussed in the methods section, based on the composition of each protein in muscle, the calculated percentage of concentration of HMM S-2, sarcoplasmic proteins, and collagen that contribute to the thermal denaturation of Peak 2 were 17.1, 66.8, and 16.1 %, respectively.

The result shows that among the three proteins, HMM S-2 was the most thermally labile with E_a of 225 kJ mol^{-1} and rate constant $k_{52°C}$ = 0.3272 min^{-1}, while collagen was found to be the most thermally stable with high E_a of 692 kJ mol^{-1} and rate constant $k_{52°C}$ = 0.0001 min^{-1}. The E_a value obtained for HMM S-2 was similar to the value reported for peak that can be attributed to HMM S-2 of DSC thermogram of isolated myofibrils (buffered in Ringer's solution and pH 5.6), which was 255 kJ mol^{-1} (Wagner & Añon, 1985). Similarly, the E_a value of thermal denaturation of sarcoplasmic protein obtained in this study, 408 kJ mol-1, was also consistent with that obtained from the round meat cut which was 406 kJ mol^{-1} (Shibata-Ishiwatari, Fukuoka, & Sakai, 2015). Furthermore, the E_a value for thermal denaturation of collagen in intact muscle in the present study (692 kJ mol^{-1}) was higher than that reported for hydrated isolated perimysium (338 kJ mol^{-1}) (Latorre et al., 2018a). Since thermal denaturation of collagen in post-mortem meat with a given pH and ionic strength is lower compared to water medium (Aktaş, 2003; Aktaş & Kaya, 2001), the higher E_a value of collagen obtained in the present study could be attributed to the presence of other proteins and cellular components in meat which might cause an increase in the thermal stability of collagen in intact muscle compared to isolated perimysium (Rabbani & Choi, 2018; Stadmiller & Pielak, 2021). Furthermore, collagen fibres contract to one-fourth of their original length during thermal denaturation. However, in intact muscle collagen can shrink only to a

certain degree due to the resistance of muscle fibres (Lepetit, 2007). Thus, the restraining of collagen in intact muscle from shrinkage due to physical effect of muscle fibres might also explain the higher thermal stability of collagen in intact muscle compared to isolated perimysium that is free to shrink during thermal treatment (Snowden, Bouton, & Harris, 1977). Interestingly, the E_a value of thermal denaturation of collagen in the present study was in agreement with the result reported by Bertola et al. (1994). Using the data of peak shear force value associated with the tenderisation process and denaturation of Peak 2 of DSC thermogram, they reported the activation energy of tenderisation process of 605 kJ mol^{-1} which is linked to Peak 2 denaturation.

Table 5. 3 Estimated fitting parameters obtained from a single first-order reaction model and three-protein model.

Parameter	Value	Unit
Single first order reaction model		
E_a	392	kJ mol^{-1}
$k_{52°C}$	0.0054	min^{-1}
R^2	0.836	
Three-protein model		
E_{aN1}	225	kJ mol^{-1}
E_{aN2}	408	kJ mol^{-1}
E_{aN3}	692	kJ mol^{-1}
$k_{52°CN1}$	0.3272	min^{-1}
$k_{52°CN2}$	0.0059	min^{-1}
$k_{52°CN3}$	0.0001	min^{-1}
N_1	0.1707	
N_2	0.6679	
N_3	0.1614	
R^2	0.926	

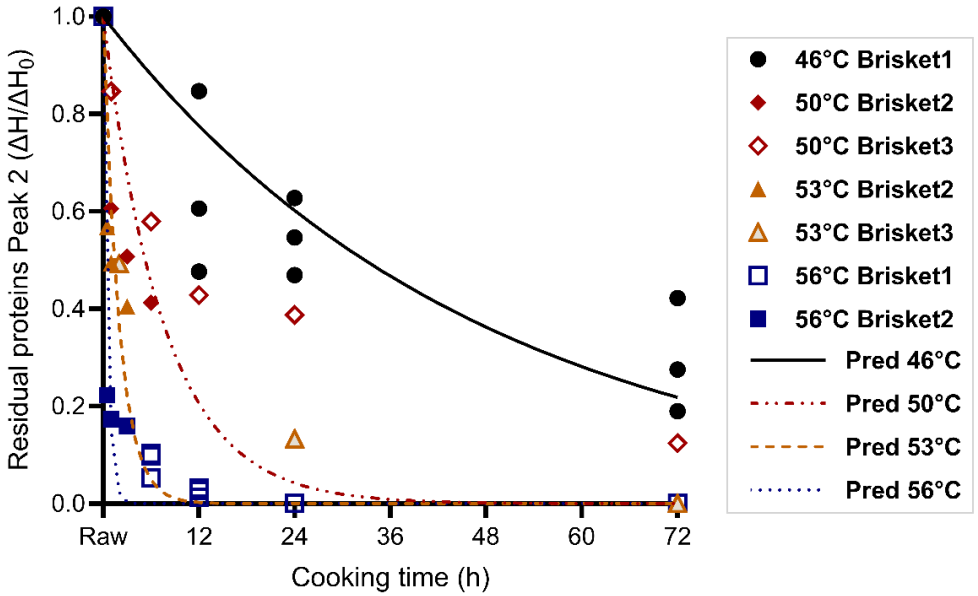

Figure 5. 2 Residual enthalpy of denaturation of Peak 2 fitted to the single first-order reaction model.

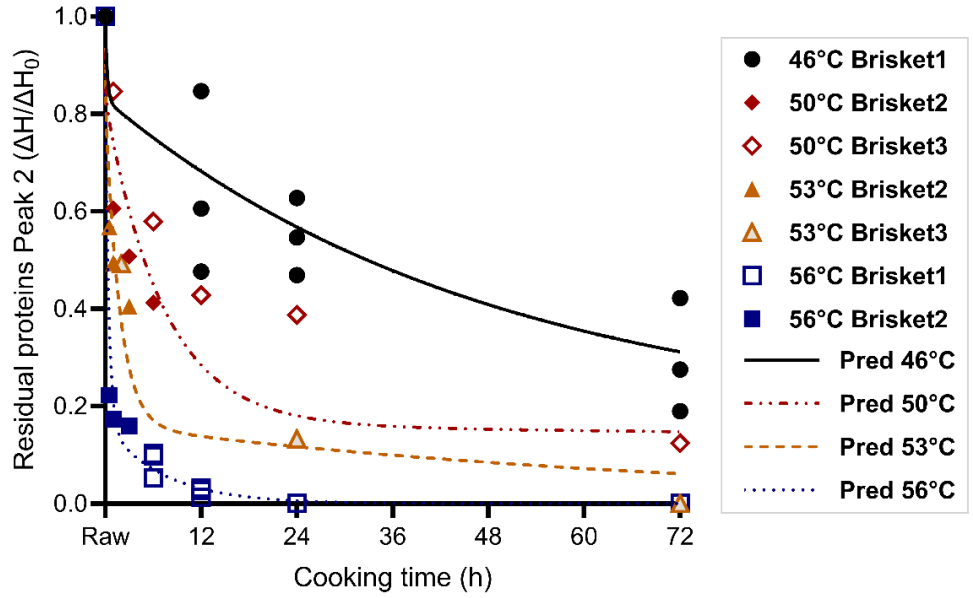

Figure 5. 3 Residual enthalpy of denaturation of Peak 2 fitted to the three-protein model.

5.3.3 Simulation of thermal denaturation of Peak 2 proteins

In order to verify the kinetics of collagen denaturation obtained from the three-protein model, the change in the fraction of denatured collagen at temperatures 46, 50, 53, 56, 60, and 70 °C was calculated using the kinetic parameters obtained from the model (Figure 5.4). No collagen denaturation was observed at 46 °C even after 72 h of cooking. At 50 °C, there was a slow increase in the amount of denatured collagen, with less than 10 % of denatured collagen observed after 72 h of cooking. This is consistent with the results of Wang et al. (2022), using fluorescent-labelled peptide that can specifically bind to denatured collagen chains, who reported that cooking at 50 °C for 4 h only produced a small amount of denatured collagen. Moreover, they also reported a marked increase in the amount of denatured collagen with increasing cooking treatment from 50 °C for 4 h to 60 °C for 4 h, where homogenous fluorescence signal was already detected in both endomysium and perimysium structures. Increasing cooking treatment to 70 °C for 4 h only, contributed to a slight increase in the fluorescence intensity which indicate that cooking treatment at 60 °C for 4 h can already produce complete collagen denaturation. The results are in agreement with the model, which show that cooking at 50 °C for 4 h only cause 0.5 % of collagen denaturation, while collagen was completely denatured after 2 h of cooking at 60 °C. It is worth noting that the results obtained in this study contradict the results reported by Latorre, Palacio, Velázquez, and Purslow (2019) which showed that cooking at 60 °C for 24 h only caused 50 % of collagen denaturation. However, this discrepancy may be attributed to different muscle (*semitendinosus*), measurement techniques (trypsin solubility) and conditions used in their study. The high elastin content in the *semitendinosus* muscle may contribute to the amount of undenatured collagen and reduce the susceptibility of denatured collagen to trypsin, thus causing underestimation of the amount of denatured collagen (Bendall, 1967).

Several studies have demonstrated that the temperature of collagen thermal denaturation (T_{max}) obtained from DSC varies with scanning rate. Decreasing the scanning rate causes the shift of T_{max} to a lower temperature due to a longer equilibration time at constant temperature (Latorre et al., 2018a; Miles, 1993). This led Miles and Bailey (1999) to propose that collagen thermal

denaturation is governed by a rate process where temperature affects the rate of collagen denaturation. Overall, this explains the results obtained in this and other studies that show collagen denaturation can occur even below its measured thermal denaturation temperature, especially under prolonged cooking times (Li et al., 2019; Wang et al., 2022).

Since pH and salt concentration affect the thermal stability of collagen, the kinetic parameters of collagen thermal denaturation obtained in this study might differ from those of meat with different pH and salt content. Aktaş and Kaya (2001) reported that decreasing the pH from 6.5 to 2.9 caused a decrease in the collagen thermal denaturation temperature (T_{max}) from 60 °C to 39 °C. Similarly, Rios-Mera, da Silva Pinto, and Contreras-Castillo (2017) reported that meat with a post-mortem pH of 5.4 – 5.8 has a T_{max} value of 65 °C, which is lower compared to 69 °C for meat with a pH of 5.8 – 6.2. Furthermore, the addition of salt such as sodium chloride (NaCl), calcium chloride ($CaCl_2$), and sodium tripolyphosphate (TPP) has also been reported to increase the thermal stability of collagen (Aktaş & Kaya, 2001; Pighin, Sancho, & Gonzalez, 2008).

Several studies have reported that cathepsin B and L are able to degrade native collagen, leading to collagen solubilisation (Burleigh et al., 1974; Kirschke et al., 1982; Maciewicz et al., 1987). Additionally, the degradation of collagen is faster in denatured collagen compared to native collagen (Shoji et al., 2014). Therefore, in this study, the kinetics of collagen thermal denaturation were determined. This information can be used in combination with the kinetics of cathepsin B and L activity to select temperature and time combinations where both the concentrations of denatured collagen and cathepsin B and L activity are high. It is expected that at this temperature and time combination, the rate of connective tissue proteolysis will be the highest, thus can produce greater collagen solubilisation, and consequently meat tenderness, either directly or upon cooking at the second-stage temperature. However, based on the comparison between the fraction of denatured collagen and cathepsin B and L activity (Figure 5.5), it appears that both processes are optimal at different temperature regimes. The marked increase in the extent of collagen denaturation occurs at 56 °C. However, at this temperature, the rate of cathepsin B and L denaturation is also high. To put it differently, at temperatures where cathepsin activity is high,

most of the collagen remains in its native state. As a result, the effect of both factors on meat tenderness will be determined individually.

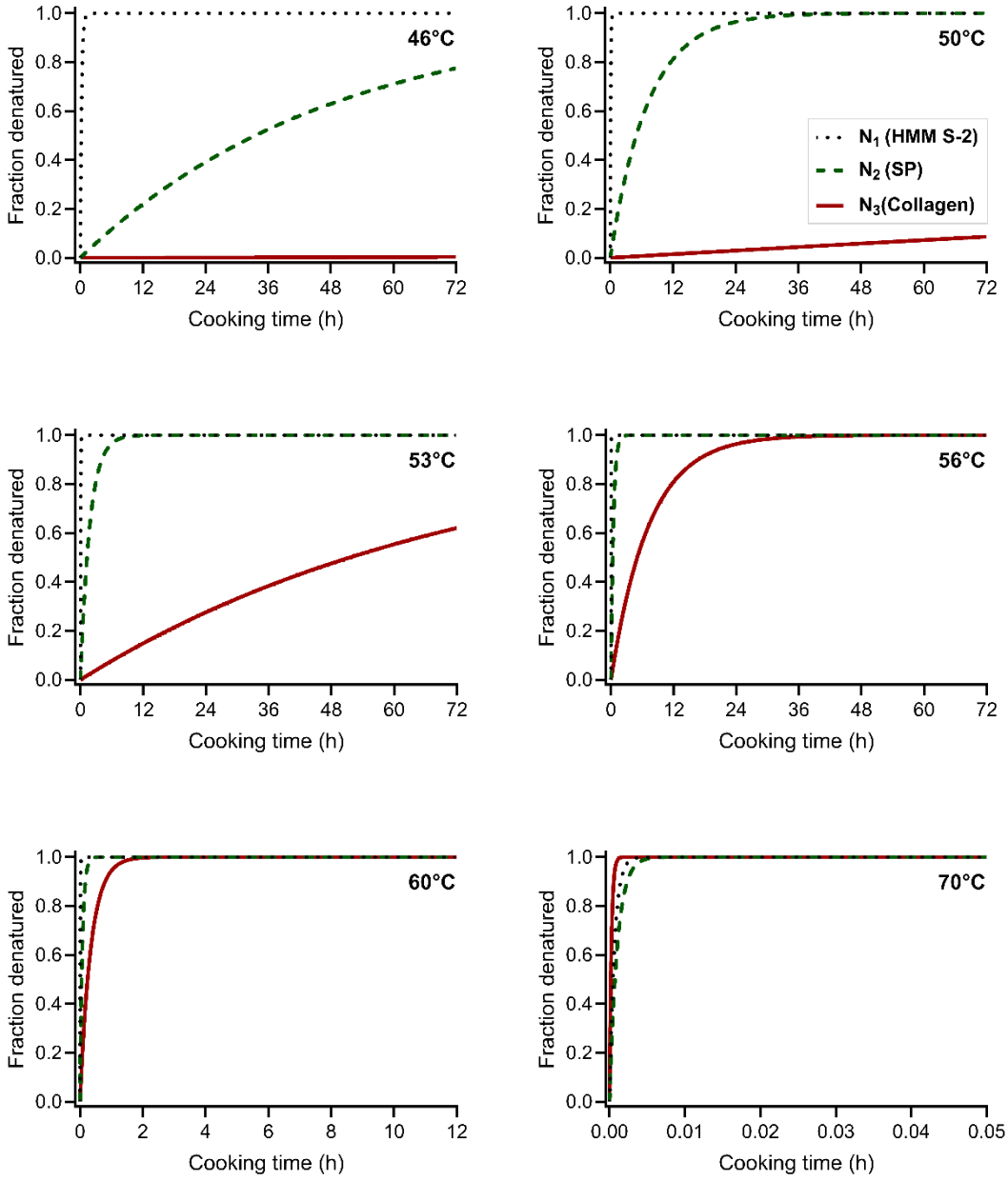

Figure 5. 4 Predicted fraction of denatured collagen at temperature 46, 50, 53, 56, 60, and 70 °C obtained from the three-protein model. Fractions of denatured HHM-S2 and sarcoplasmic proteins (SP) were also presented as comparison.

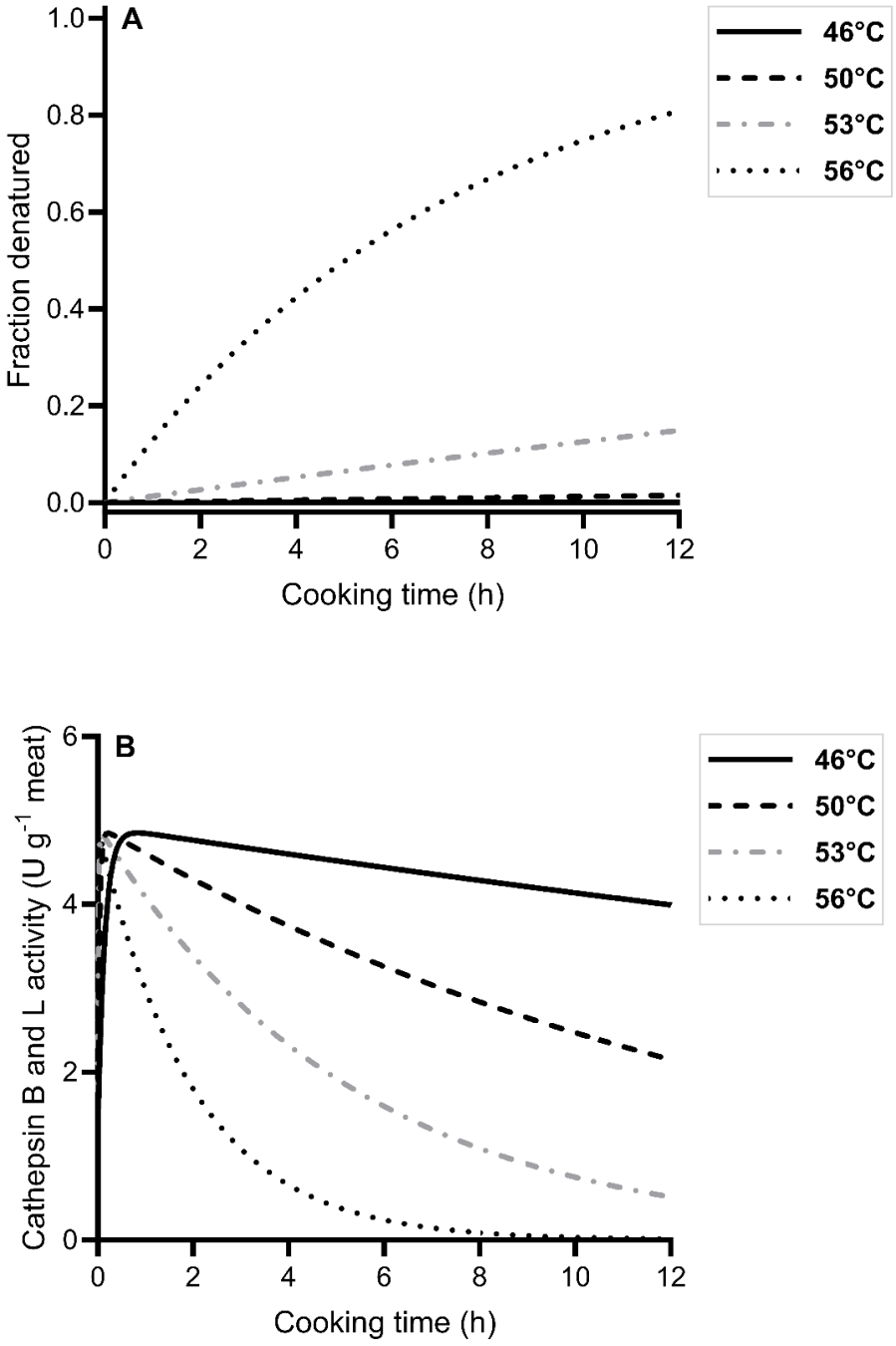

Figure 5. 5 A. Fraction of denatured collagen (three-protein model) and **B.** Cathepsin B and L activity (Model 1) at 46, 50, 53, and 56 °C.

5.4 Conclusion

In this study, the kinetics of the thermal denaturation of collagen were modeled using the thermal denaturation data of Peak 2 generated by DSC. The three-protein model showed better and more satisfactory fitting compared to a single first-order reaction with R^2 values of 0.926 and 0.836, respectively. Based on the three-protein model, the activation energies for the thermal denaturation of HMM S-2, sarcoplasmic proteins, and collagen were found to be 225, 408, and 692 kJ mol^{-1}, respectively.

The results in the previous chapter (Chapter 4) show precooking at 46°C, which represents high cumulative cathepsin B and L activity, does not promote tenderisation. One of the reasons could be that the tenderisation due to myofibrillar proteolysis is masked by connective tissue toughness due to the lack of collagen solubilisation at the selected second-stage temperature of 71 °C(Tc). Thus, the effects of precooking at varying levels of cumulative cathepsin B and L activity and degree of collagen denaturation and subsequently cooking at temperature treatments that can promote collagen solubilisation were investigated in Chapter 6.

CHAPTER 6 Two-stage *sous vide* cooking

6.1 Introduction

Sous vide is a culinary technique that involves cooking foods inside heat-stable vacuum-sealed pouches under precisely controlled temperature and time (Baldwin, 2012). This cooking technique has gained interest in restaurants, homes, and the food industry due to its ability to ensure greater consistency in end products and to tenderise tough meat cuts (Baldwin, 2012; Christensen et al., 2011; Fareh, 2018; Gámbaro et al., 2023; Naqvi et al., 2021). The tenderisation of meat during *sous vide* cooking is attributed, but not limited to, collagen solubilisation (Dominguez-Hernandez et al., 2018). Several studies have reported that tough meat cuts, such as shank and eye of round, characterised by a high amount of collagen content, require longer cooking time or higher cooking temperature to become tender, in comparison to tender meat with low collagen content, such as tenderloin and top loin (Christensen et al., 2011; Gámbaro et al., 2023). This aligns with the recommended *sous vide* cooking conditions by chefs for tough meat cuts, which typically involve cooking at low temperatures (50 – 65 °C) for a prolonged time (24 to 72 h) or at high temperatures (80 – 90 °C) for a shorter time (12 to 24 h) (Baldwin, 2021; Myhrvold, Young, & Bilet, 2011). The shorter cooking time required at higher temperatures is attributed to the faster rate of collagen solubilisation (Fareh, 2018). From the industrial perspective, implementing high-temperature short-time *sous vide* cooking to process tough meat cut such as beef brisket is preferable for achieving higher product throughput and better operational efficiency. However, it is important to note that high temperatures also result in increased cooking loss, attributed to cooperative shrinkage between collagen and muscle fibres and proteins aggregation (Tornberg, 2005). Additionally, at high temperatures, the potential benefits of proteolytic activity from endogenous enzymes, such as cathepsin B and L, which can be achieved at low-temperature cooking, are compromised due to a faster deactivation rate.

In order to obtain the benefits of both cooking temperature regimes, several studies have explored the use of two-stage cooking. This involves initial low-temperature cooking (first stage) followed

by high-temperature cooking (second stage), which has been shown to improve tenderness and cooking yield of meat compared to one-stage high-temperature cooking (Ismail et al., 2019; Obuz et al., 2003; Pietrasik, Dhanda, Pegg, & Shand, 2005; Powell et al., 2000; Walsh et al., 2010; Yao et al., 2023). However, much uncertainty still exists about the temperature-time combination during the first-stage cooking that can promote greater tenderisation and reduction in cooking loss. Furthermore, the effect of variation in cathepsin B and L activity and the degree of collagen denaturation during the first-stage cooking on cooked meat tenderness, cooking loss, and collagen solubilisation has not been fully understood, thus requiring further investigation. Moreover, previous studies have primarily focused on temperatures between 70 and 75 °C for the second-stage treatment, excluding temperatures commonly used for *sous vide* cooking of tough meat cuts (Obuz et al., 2003; Pietrasik et al., 2005; Powell et al., 2000; Walsh et al., 2010). Thus, the objective of this study is to evaluate the potential of two-stage cooking in comparison to one-stage cooking to improve the tenderness and cook yield of beef brisket, under varied temperature and time combinations during the first-stage and second-stage cooking. Additionally, the study seeks to understand how the variation in cumulative cathepsin B and L activity, and the degree of collagen denaturation obtained during the first-stage cooking contributes to the observed results. This study was divided into two sections: (1) Varied the first stage, involving different combinations of temperature and time during the first-stage cooking, followed by two fixed second-stage conditions representing *sous vide* cooking commonly used in the kitchen (56 °C for 48 h) and in the food industry (80 °C for 8 h); (2) Varied the second stage, consisting of one fixed first-stage condition followed by different combinations of temperature and time during the second-stage cooking.

6.2 Materials and methods

6.2.1 Sample

Experiment 1

A total of nine pairs of cold-boned beef briskets from mix British cattle (24-36 months, six heifers and three steers) were obtained from a meat processing plant (Taylor Preston Limited) in two batches (one week apart).

Experiment 2

A total of eight pairs of cold-boned beef briskets from mix British heifers (24-36 months) were obtained from a meat processing plant (Taylor Preston Limited).

6.2.2 Sample preparation

The sample was prepared as described in Chapter 4, section 4.2.2. Briefly, only the middle part of the brisket was used: the head, tail, and left and right side (offcuts) were saved for pH measurements. Subsequently, the briskets were cut across the muscle fibres into ten steaks with 2.5 cm thickness. The external fat and connective tissue were trimmed off from each steak. The steaks were placed inside heat-stable vacuum bag (EVB vacuum bag, 95 μm thick), vacuum-packed to 15 mbar using a vacuum packing machine (Multivac New Zealand Ltd), weighed, and stored at 4 °C. For each pair of brisket, the first steak was used for raw meat analysis (total collagen content) while the rest of the sample were assigned for different heat treatments. The sample was *sous vide* cooked at 8 days and 11 days post-mortem (dry aging), for Experiment 1 and 2, respectively.

6.2.3 Experimental design

Experiment 1 **(Varied first stage and fixed second stage at 56 °C for 48 h + 71 °C (Tc) and 80 °C for 8 h).**

One slice from each animal was assigned for 71 °C (Tc) *sous vide* cooking for standard tenderness evaluation (AMSA, 2016). The rest of the samples from different animals, batches, and locations on the muscle was randomly assigned to one of 49 treatments (Figure 6.1): **(1) Stage 1** at 46, 50, 53, and 56 °C for 0, 2, 6, and 12 h; **(2) 56_48h**: Two-stage 56_48h (first stage as describes in A and second stage at 56 °C for 48 h followed by at 71 °C (Tc)) and One-stage 56_48h (directly cooked at 56 °C for 48 h followed by at 71 °C (Tc)) and; **(3) 80_8h**: Two-stage 80_8h (first stage as describes in A and second stage at 80 °C for 8 h) and One-stage 80_8h (directly cooked at 80 °C for 8 h).

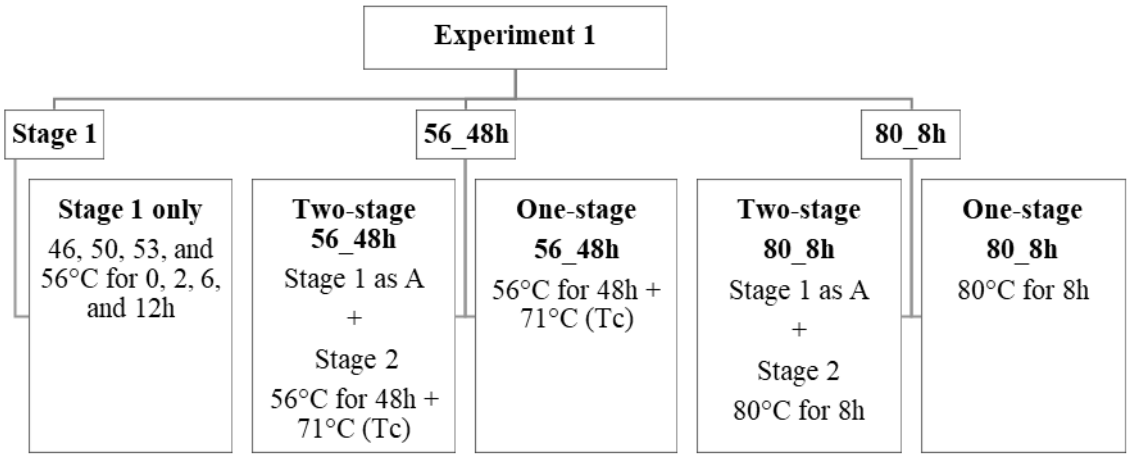

Figure 6. 1 Schematic diagram of Experiment 1.

Experiment 2 **(Varied second stage and fixed first stage at 56 °C for 6h)**

One slice from each animal was assigned for 71 °C (Tc) *sous vide* cooking for standard tenderness evaluation (AMSA, 2016). The rest of the samples from different animals and locations on the muscle was randomly assigned to one of 44 treatments (Figure 6.2): **(1)** Conventional *sous vide* cooking at 60, 70, 80, and 90 °C for different time periods (2, 6, 8, 12, 14, 20, and 26 h), **(2)** Two-

stage *sous vide* cooking which involve first stage at 56 °C for 6 h and second stage at 60, 70, 80, and 90 °C for different time periods (0, 2, 6, 8, 14, and 20 h).

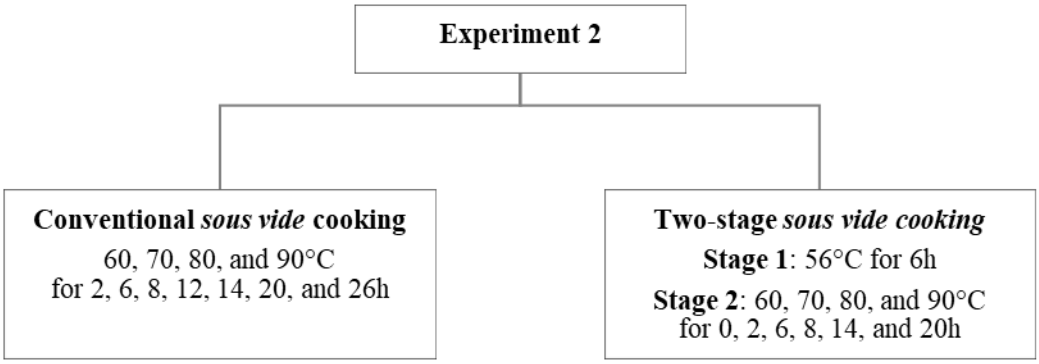

Figure 6. 2 Schematic diagram of Experiment 2.

For 71 °C (Tc) treatment, the samples were *sous vide* cooked at 71 °C water bath until the core temperature reaches 71 °C. For 0 h treatment the samples were *sous vide* cooked at the designated temperature until the core reaches the temperature (no holding time). While for the rest of cooking time treatments, once the core temperature reaches the designated temperature, the sample was held at that temperature according to the designated time. Cooking was stopped by immersing samples in ice water baths for 15 mins. The samples were used for shear force, cooking loss, pH, and collagen analysis. Sample for collagen analysis were stored at -20 °C until further analysis. Meanwhile, samples for shear force, cooking loss, and pH measurement were stored at 4 °C and analysed within 24 h.

6.2.4 Cooking treatment

The sample was *sous vide* cooked as described in Chapter 4, section 4.2.5.

6.2.5 pH measurement

pH measurement was carried out as described in Chapter 4, 4.2.6 except for the pH of cooked meat sample, the measurement was carried out using the cook loss fraction.

6.2.6 Shear force measurement

Shear force measurement was conducted following the procedures outlined in Chapter 4, 4.2.7, with the exception that a blade thickness of 3 mm was used in this study. Specifically, square cross-section cores (10 × 10 mm) were removed parallel to the muscle fibre using a two-bladed knife as a guide and a sharp knife to cut the sample (Silva et al., 2015). Four to six cores were obtained for each meat sample. Shear force measurements were performed using texture analyser (Model TA-XTPlus, Stable Micro Systems Ltd, UK) with 50 kg load cell and triangular cutting (V-shaped, 3 mm thickness) blade attachment (HDP/WBV, Stable Micro Systems Ltd, UK). The central portion of the sample was positioned under the blade and sheared perpendicular to the muscle fibre direction with crosshead speed 200 mm/ min (AMSA, 2016).

6.2.7 Cooking loss

Cooking loss measurement was carried out as described in Chapter 4, 4.2.8.

6.2.8 Collagen determination

Collagen determination was carried out as described in Chapter 4, 4.2.11 and 4.2.12.

6.2.9 Statistical analysis

Statistical analysis was performed using SAS® Studio 3.8 (SAS Institute, Cary, NC, USA). All data were tested for homogeneity of variance using Levene's test. The SAS PROC MIXED procedure was used to evaluate the effect of heat treatments on peak shear force, cooking loss, solubilised collagen in cook loss per total collagen (SC), and pH. For **Experiment 1**: to compare significant different in shear force value, cooking loss, and SC between treatment Stage 1 only, 56_48h, and 80_8h, a model with temperature, time, treatment, and their interaction (up to three-way interaction) were specified as fixed effects, animal, batch, and sex as random effects, and location within animal as repeated effect. For this model, the data of One-stage 56_48h and One-stage 80_8h were excluded. Furthermore, to compare significant different in shear force value,

cooking loss, and SC within treatment Stage 1 only, 56_48h, and 80_8h (the three treatments were analysed separately), another model was used where temperature, time, and their interaction were specified as fixed effects, animal, batch, and sex as random effects, and location within animal as repeated effect. In order to include One-stage 56_48h (temp=56°C, time=0 h) and One-stage 80_8h (temp=80°C, time=0 h) in the analysis of treatment 56_48h and 80_8h, respectively, another fixed effect term (two-stage-vs-one-stage) was added to the model and other fixed effects were nested within this effect (Naqvi et al., 2022).

For **Experiment 2**: cooking procedure (conventional vs. two-stage), temperature, time, and their interaction (up to three-way interaction) were specified as fixed effects, animal as random effects, and location within animal as repeated effect. When fixed effects were significant ($P < 0.05$), multiple comparison were performed using the Tukey procedures ($P < 0.05$ indicated significance). Data that did not fulfil the assumption of equal variance were log (shear force) and square root transformed (SC) before analysed. The data were then back transformed to present the result.

Additionally, the comparison of the characteristics of raw meat and meat cooked at 71 °C (Tc) was conducted between Chapter 4 and Chapter 6 (Experiment 1), as well as between Experiment 1 and Experiment 2 in Chapter 6. This analysis was performed using the SAS® Studio 3.8 t-Test procedure. Furthermore, the SAS PROC CORR procedure was used to determine Pearson correlation coefficients between shear force values and solubilised collagen in cook loss per total collagen (%).

All figures in this study were created using GraphPad Prism v9.4.0. The technical replicates were first averaged, and then the values were reported as the mean of sample replicates (n=3) ± standard error of mean (SEM), unless specified otherwise.

6.3 Results and discussion

6.3.1 Experiment 1 (Varied first stage)

6.3.1.1 Beef brisket characteristic

Table 6. 1 Characteristic of raw and *sous vide* cooked beef brisket at 71 °C (Tc).

Attribute	Mean ± SEM
Raw brisket	
pH	5.49 ± 0.02
Total collagen (mg g^{-1} meat)	9.0 ± 0.5
***Sous vide* cooked brisket at 71 °C (Tc)**	
Peak shear force (N)	72.5 ± 2.1
Cooking loss (% w/w)	26.8 ± 0.8
Solubilised collagen in cook loss per total collagen (%)	1.48 ± 0.15

Tc (the core temperature of meat reaches the applied temperature).
n = 9 animal replicates.

Table 6.1 described the properties of raw meat and meat *sous vide* cooked at 71 °C (Tc). The pH value of raw meat used in this study is in the range of normal post-rigor pH value of beef. The value of total collagen in this study is significantly lower (P = 0.0166) and solubilised collagen (P = 0.0221) and cooking loss (P = 0.0030) is significantly higher than that of the brisket used in Chapter 4. This could be due to the different breed and aging time (8 vs. 4 days) used in this study. The higher cooking loss and solubilised collagen with extended aging has been reported in several studies (Colle et al., 2016; Modzelewska-Kapituła, Kwiatkowska, Jankowska, & Dąbrowska, 2015; Purslow, Oiseth, Hughes, & Warner, 2016; Starkey, Geesink, Oddy, & Hopkins, 2015; Vaskoska et al., 2020). The peak shear force values could not be compared because different blade specifications were used in these studies.

6.3.1.2 pH

Table 6. 2 pH value (means ± SEM) of raw and *sous vide* cooked beef brisket at 46, 50, 53, and 56 °C for 0, 2, 6, and 12 h. Different letters (a-e) within the same column statistically indicate the significant differences ($p < 0.05$).

Temperature (°C)	Time (h)	pH value
Raw	–	5.49 ± 0.02^{a}
46	0	5.69 ± 0.03^{b}
	2	5.70 ± 0.02^{bcd}
	6	5.73 ± 0.01^{bcde}
	12	5.80 ± 0.02^{bcdef}
50	0	5.72 ± 0.04^{bc}
	2	5.76 ± 0.04^{bcdef}
	6	5.85 ± 0.01^{cdef}
	12	5.81 ± 0.01^{bcdef}
53	0	5.78 ± 0.02^{bcdef}
	2	5.77 ± 0.01^{bcdef}
	6	5.83 ± 0.03^{cdef}
	12	5.86 ± 0.03^{ef}
56	0	5.76 ± 0.01^{bcdef}
	2	5.86 ± 0.02^{def}
	6	5.89 ± 0.02^{ef}
	12	5.91 ± 0.03^{f}
P value		<.0001

Table 6.2 shows the pH value of raw meat and meat cooked at low-temperature cooking (treatment A). There is a significant increase in pH value of cooked meat compared to raw meat. Additionally, there is a significant higher pH value of meat cooked at 56 °C for prolonged time (6 and 12 h) compared to meat cooked at 46 °C for a shorter time (0 and 2 h). Similar result has been reported by Laakkonen, Wellington, and Sherbon (1970) , Kaur et al. (2020), and Naqvi et al. (2022) that shows a significant increase in pH value of meat with cooking which attributed to a decrease in available acidic group in meat due to protein denaturation and aggregation (Poulter, Ledward, Godber, Hall, & Rowlands, 1985).

6.3.1.3 Peak shear force

Table 6. 3 Peak shear force value (N) (means ± SEM) of beef brisket *sous vide* cooked using Stage 1 only, 56_48h (two-stage and one-stage), and 80_8h (two-stage and one-stage) cooking conditions. Different lowercase letters (a-e) within the same column and capital letter (A-C) within the same row statistically indicate the significant differences ($p < 0.05$).

Temperature	Time	Treatment		
Stage 1 (°C)	Stage 1 (h)	**Stage 1 only**	**56_48h**	**80_8h**
46	0	$78.4 \pm 0.7_{abcd}^{A}$	31.2 ± 6.5^{B}	$66.4 \pm 4.4_{ab}^{A}$
	2	$100.2 \pm 13.1_{ab}^{A}$	36.9 ± 10.5^{C}	$53.7 \pm 3.7_{abcde}^{B}$
	6	$104.0 \pm 0.9_{ab}^{A}$	32.9 ± 2.3^{B}	$46.4 \pm 5.1_{abcde}^{B}$
	12	$85.3 \pm 3.8_{abcd}^{A}$	30.1 ± 4.5^{C}	$46.3 \pm 4.6_{bcde}^{B}$
50	0	$69.1 \pm 3.8_{bcd}^{A}$	34.0 ± 3.4^{B}	$52.5 \pm 3.2_{abcde}^{A}$
	2	$88.2 \pm 6.0_{abc}^{A}$	26.9 ± 5.1^{C}	$54.7 \pm 0.4_{abcd}^{B}$
	6	$88.8 \pm 9.2_{abcd}^{A}$	29.4 ± 3.2^{C}	$50.2 \pm 5.0_{abcde}^{B}$
	12	$104.4 \pm 8.4_{ab}^{A}$	26.1 ± 5.7^{C}	$47.3 \pm 1.4_{de}^{B}$
53	0	$78.8 \pm 8.4_{abcd}^{A}$	30.1 ± 5.0^{B}	$60.2 \pm 0.6_{abd}^{A}$
	2	$77.6 \pm 1.9_{abcd}^{A}$	39.0 ± 8.6^{B}	$50.8 \pm 3.6_{abcde}^{B}$
	6	$104.4 \pm 13.0_{ab}^{A}$	38.7 ± 3.6^{B}	$50.2 \pm 2.8_{abcde}^{B}$
	12	$89.6 \pm 5.7_{abcd}^{A}$	29.2 ± 2.5^{C}	$47.0 \pm 4.4_{bcde}^{B}$
56	0	$107.8 \pm 6.0_{ab}^{A}$	–	$62.1 \pm 2.5_{abc}^{B}$
	2	$110.5 \pm 8.5_{a}^{A}$	36.1 ± 3.1^{C}	$56.4 \pm 1.3_{abcd}^{B}$
	6	$62.6 \pm 4.6_{cd}^{A}$	30.3 ± 4.3^{B}	$44.3 \pm 1.8_{cde}^{A}$
	12	$60.9 \pm 7.3_{d}^{A}$	28.6 ± 4.5^{C}	$41.0 \pm 1.2_{e}^{B}$
One-stage 56_48h		–	36.5 ± 3.9	–
One-stage 80_8h		–	–	64.2 ± 6.6^{a}
P value within treatment				
Temperature		0.1159	0.3411	0.6407
Time		0.0792	0.1624	<.0001
Temperature x time		<.0001	0.4902	0.2071

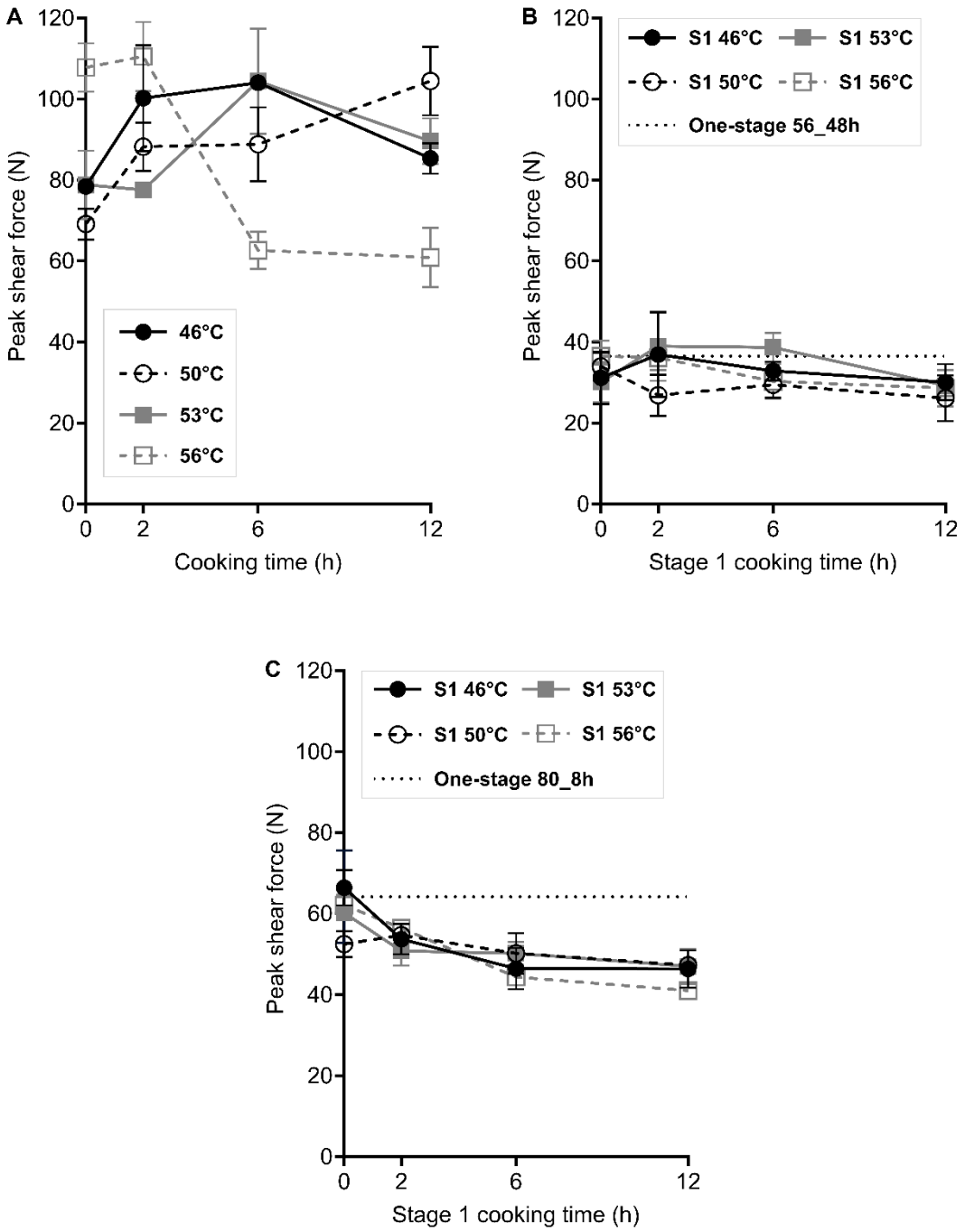

Figure 6. 3 Representation of result described in Table 6.3. A) Stage 1 only, B) 56_48h (two-stage and one-stage), and C) 80_8h (two-stage and one-stage). S1 refer to Stage 1 temperature. Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol).

6.3.1.4 Cooking loss

Table 6. 4 Cooking loss (%) (means ± SEM) of beef brisket *sous vide* cooked using Stage 1 only, 56_48h (two-stage and one-stage), and 80_8h (two-stage and one-stage) cooking conditions. Different lowercase letters (a-c) within the same column and capital letter (A-C) within the same row statistically indicate the significant differences ($p < 0.05$).

Temperature	Time	Treatment		
Stage 1 (°C)	Stage 1 (h)	**Stage 1 only**	**56_48h**	**80_8h**
46	0	$10.9 \pm 2.0_{a}^{A}$	32.9 ± 0.9^{B}	43.5 ± 1.0^{C}
	2	$15.3 \pm 1.0_{ab}^{A}$	29.5 ± 1.2^{B}	43.8 ± 1.4^{C}
	6	$17.2 \pm 1.0_{ab}^{A}$	31.7 ± 0.2^{B}	46.2 ± 1.1^{C}
	12	$17.0 \pm 2.5_{b}^{A}$	31.1 ± 0.5^{B}	44.1 ± 0.1^{C}
50	0	$15.5 \pm 1.2_{ab}^{A}$	30.9 ± 1.2^{B}	44.8 ± 1.3^{C}
	2	$18.5 \pm 0.6_{b}^{A}$	31.7 ± 1.8^{B}	44.9 ± 1.0^{C}
	6	$18.6 \pm 1.1_{bc}^{A}$	32.9 ± 1.2^{B}	46.9 ± 0.6^{C}
	12	$21.8 \pm 1.1_{bc}^{A}$	34.8 ± 1.7^{B}	44.9 ± 0.6^{C}
53	0	$15.9 \pm 0.1_{ab}^{A}$	33.9 ± 1.5^{B}	44.8 ± 0.4^{C}
	2	$21.7 \pm 0.7_{bc}^{A}$	31.4 ± 1.1^{B}	44.5 ± 0.8^{C}
	6	$19.4 \pm 0.7_{bc}^{A}$	30.8 ± 1.5^{B}	44.8 ± 0.6^{C}
	12	$20.3 \pm 0.4_{bc}^{A}$	32.9 ± 0.5^{B}	45.4 ± 0.5^{C}
56	0	$19.5 \pm 0.4_{bc}^{A}$	–	43.3 ± 2.2^{B}
	2	$18.1 \pm 1.1_{b}^{A}$	31.5 ± 2.0^{B}	44.3 ± 0.3^{C}
	6	$23.8 \pm 1.8_{c}^{A}$	32.7 ± 0.9^{B}	41.8 ± 1.3^{C}
	12	$24.9 \pm 1.3_{c}^{A}$	33.3 ± 0.9^{B}	42.6 ± 1.9^{C}
One-stage 56_48h		–	32.0 ± 0.8	–
One-stage 80_8h		–	–	44.0 ± 0.1
P value within treatment				
Temperature		<.0001	0.5955	0.0186
Time		<.0001	0.2616	0.8179
Temperature x time		0.1781	0.4890	0.3989

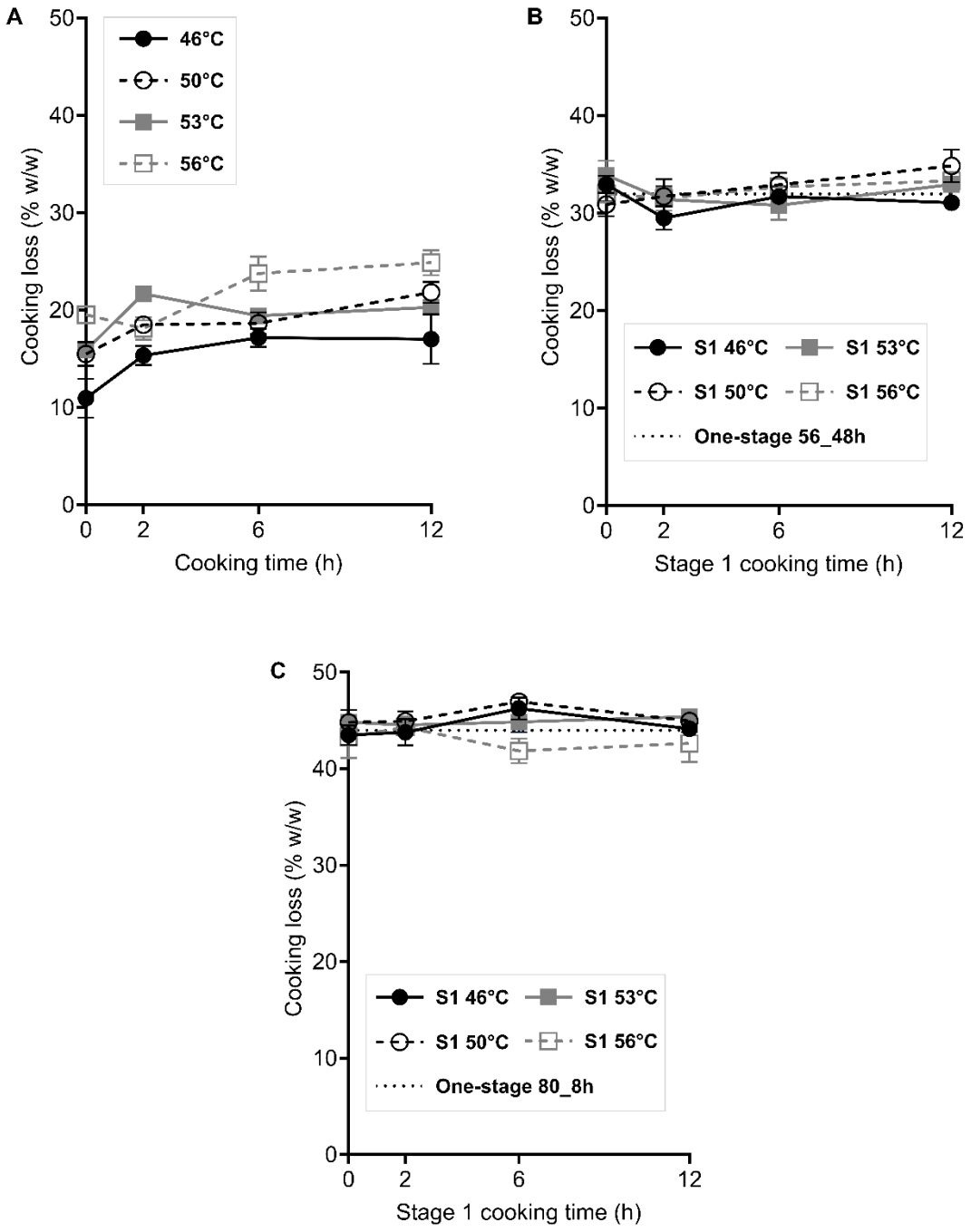

Figure 6. 4 Representation of result described in Table 6.4. A) Stage 1 only, B) 56_48h (two-stage and one-stage), and C) 80_8h (two-stage and one-stage). S1 refer to Stage 1 temperature. Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol).

6.3.1.5 Solubilised collagen in cook loss

Table 6. 5 Solubilised collagen in cook loss per total collagen (%) (means ± SEM) of beef brisket *sous vide* cooked using Stage 1 only, 56_48h (two-stage and one-stage), and 80_8h (two-stage and one-stage) cooking conditions. Different lowercase letters (a-c) within the same column and capital letter (A-C) within the same row statistically indicate the significant differences ($p < 0.05$).

Temperature	Time	Treatment		
Stage 1 (°C)	Stage 1 (h)	**Stage 1 only**	**56_48h**	**80_8h**
46	0	$0.5 \pm 0.1_{a}^{A}$	6.7 ± 0.6^{B}	8.4 ± 0.4^{C}
	2	$0.7 \pm 0.1_{abc}^{A}$	6.4 ± 2.0^{B}	7.2 ± 0.7^{B}
	6	$1.1 \pm 0.1_{abcd}^{A}$	5.2 ± 0.4^{B}	8.7 ± 1.1^{C}
	12	$1.0 \pm 0.2_{abcd}^{A}$	5.4 ± 0.6^{B}	8.4 ± 0.8^{C}
50	0	$0.7 \pm 0.1_{abc}^{A}$	4.4 ± 0.5^{B}	8.4 ± 0.7^{C}
	2	$1.0 \pm 0.0_{abcd}^{A}$	6.9 ± 1.4^{B}	7.5 ± 0.3^{C}
	6	$1.0 \pm 0.0_{abcde}^{A}$	6.5 ± 0.9^{B}	12.9 ± 1.5^{C}
	12	$1.5 \pm 0.0_{cde}^{A}$	6.0 ± 1.2^{B}	8.2 ± 0.3^{C}
53	0	$0.6 \pm 0.1_{ab}^{A}$	8.2 ± 1.3^{B}	10.3 ± 1.1^{B}
	2	$1.1 \pm 0.0_{abcde}^{A}$	5.6 ± 0.6^{B}	8.6 ± 1.6^{C}
	6	$1.2 \pm 0.0_{bcde}^{A}$	3.9 ± 0.2^{B}	10.4 ± 0.6^{C}
	12	$1.4 \pm 0.1_{cde}^{A}$	6.9 ± 0.6^{B}	11.4 ± 0.7^{C}
56	0	$1.1 \pm 0.0_{abcd}^{A}$	–	8.5 ± 0.9^{B}
	2	$1.2 \pm 0.2_{bcde}^{A}$	5.2 ± 1.1^{B}	10.9 ± 0.6^{C}
	6	$1.8 \pm 0.3_{de}^{A}$	7.9 ± 2.7^{B}	9.8 ± 2.1^{B}
	12	$2.0 \pm 0.4_{e}^{A}$	7.6 ± 1.9^{B}	9.8 ± 1.8^{B}
One-stage 56_48h		–	5.5 ± 1.5	–
One-stage 80_8h		–	–	8.3 ± 1.2
P value within treatment				
Temperature		<.0001	0.4195	0.3824
Time		<.0001	0.6666	0.0914
Temperature x time		0.9036	0.5435	0.1187

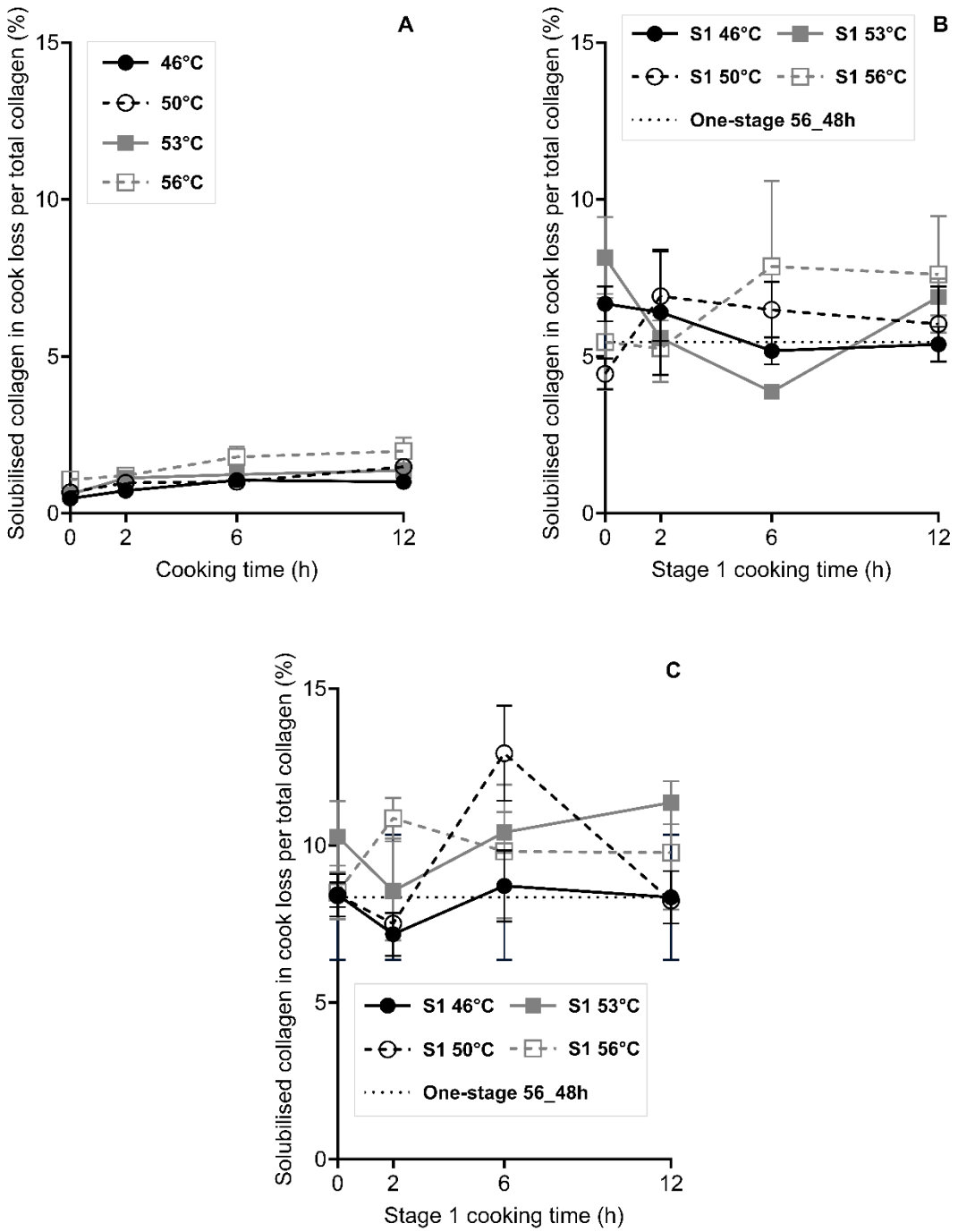

Figure 6. 5 Representation of result described in Table 6.5. A) Stage 1 only, B) 56_48h (two-stage and one-stage), and C) 80_8h (two-stage and one-stage). S1 refer to Stage 1 temperature. Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol).

Table 6.3 shows the shear force value of beef brisket *sous vide* cooked using different cooking treatments (the data was illustrated in Figure 6.3). There was three-way interaction between cooking treatment, temperature, and time (P = 0.0061) on the shear force value of meat. Meat cooked using Stage 1 cooking alone exhibits a high shear force value ranging between 61 and 108 N, indicating that Stage 1 cooking alone was insufficient to tenderise the meat. Additionally, the shear force value of the meat cooked with Stage 1 cooking alone was consistently higher than that of meat cooked using two-stage 56_48h treatment and two-stage 80_8h treatment (excluding the treatment where the Stage 1 cooking was performed at 46 to 56 °C for 0h and at 56 °C for 6 h). This suggests that combining Stage 1 cooking with low-temperature long-time treatment (56_48h) or high-temperature short-time treatment (80_8h) is necessary to reduce the shear force value of beef brisket. Notably, the two-stage 56_48h treatment consistently resulted in greater tenderness than that two-stage 80_8h treatment (excluding treatment where the Stage 1 cooking was performed at 46 °C for 6 h, 53 °C for 2 h, and 6 h), with the shear force value in the range of 28.6 – 39.0 N compared to 41.0 – 66.4 N for 80_8h treatment.

Regarding the comparison between one-stage and two-stage cooking, there was no significant difference in shear force values (Table 6.3) for the 56_48h treatment, suggesting that one-stage cooking at the 56_48h treatment alone achieved sufficient tenderisation without Stage I treatment. By contrast, the meat cooked using the one-stage 80_8h treatment exhibits a higher shear force value (64.2 N) than two-stage 80_8h treatment. Particularly, where Stage 1 was performed at the temperature of 56 °C for 6 h and at 46, 50, 53, and 56 °C for 12 h, it significantly reduces the shear force value to a range of 41.0 – 47.3 N. This suggests the benefits of employing a two-stage cooking approach for high-temperature short-time *sous vide* cooking (80_8h).

Table 6.4 shows the cooking loss of beef brisket *sous vide* cooked using different cooking treatments (the data was illustrated in Figure 6.4). There was a significant interaction between cooking treatment and time (P = 0.0004) and also between cooking treatment and temperature (P <.0001) on cooking loss. Meat cooked using two-stage 80_8h treatment exhibits consistently higher cooking loss (41.8 – 46.9 %) than meat cooked using two-stage 56_48h treatment (29.5 –

34.8 %). Meanwhile, meat cooked with Stage 1 alone demonstrates the lowest cooking loss (10.9 – 24.9 %) across all temperature-time combinations. Additionally, the results indicate no significant difference in cooking loss between two-stage and one-stage cooking for both 58_48h and 80_8h treatments, suggesting that two-stage cooking did not contribute to a reduction in cooking loss.

Table 6.5 shows the solubilised collagen in beef brisket *sous vide* cooked using different cooking treatments (the data was illustrated in Figure 6.5). There was a significant three-way interaction between cooking treatment, temperature, and time (P = 0.0084) on solubilised collagen. Meat cooked using two-stage 80_8h treatment exhibits consistently higher solubilised collagen (7.2 – 12.9 %) than meat cooked using two-stage 56_48h treatment (3.9 – 8.2 %) (excluding treatment where the Stage 1 cooking was performed at 46 °C for 2 h, 53 °C for 0 h, and 56 °C for 6 h and 12 h). Meanwhile, meat cooked with Stage 1 cooking alone demonstrates the lowest solubilised collagen (0.5 – 2 %). Similar to cooking loss, the results reveal no significant difference in solubilised collagen between two-stage and one-stage cooking for both 56_48h and 80_8h treatments. This suggests that two-stage cooking did not contribute to an increase in solubilised collagen.

In the earlier chapter (Figure 4.12, Chapter 4), it has been shown that cooking of meat to a centre temperature of 71 °C following cooking at 56 °C even for a shortest cooking time (0 h), resulted in complete protein denaturation. This suggests that meat that was cooked using two-stage 56_48h and 80_8h treatment has undergone complete protein denaturation. Thus, the higher cooking loss in two-stage 80_8h treatment compared to two-stage 56_48h treatment, could be attributed to factors other than protein denaturation, such as a greater myofibrillar protein oxidation and aggregation. Several studies have reported that the oxidation and aggregation of myofibrillar proteins are promoted at higher cooking temperatures. (Promeyrat, Daudin, & Gatellier, 2013; Promeyrat, Gatellier, et al., 2010; Traore et al., 2012; Xu et al., 2023). Protein oxidation and subsequent aggregation can reduce the water-holding capacity of meat (Estévez, 2011). This is consistent with the results reported by Traore et al. (2012) that demonstrated that cooked meat

with a greater degree of protein oxidation and aggregation experiences higher cooking loss. The higher shear force value of meat cooked using two-stage 80_8h treatment compared to 56_48h might also be due to a greater degree of protein aggregation at a higher temperature treatment, as protein aggregation has been associated with the toughening of the myofibrillar structure (Martens et al., 1982). Furthermore, even though the two-stage 80_8h treatment has higher solubilised collagen, it also exhibits higher shear force value than two-stage 56_48h treatment. This suggests that the improvement in meat tenderness is a dynamic balance between collagen solubilisation and myofibrillar toughening.

In this study, the improvement in meat tenderness in two-stage compared to one stage cooking for 80_8h treatment was observed in meat cooked using Stage 1 cooking at 46, 50, and 53 °C for 12 h and at 56 °C for 6 and 12 h. Based on the data from Figure 3.8 (Chapter 3) and Figure 5.4 (Chapter 5), Stage 1 cooking at 46, 50, and 53 °C for 12 h is characterised by high cumulative cathepsin B and L activity, low or negligible collagen denaturation, and a high degree of sarcoplasmic protein denaturation (only for the 50 and 53 °C treatments for 12 h). In contrast, Stage 1 cooking at 56 °C for 6 and 12 h is characterised by low cumulative cathepsin B and L activity and a high degree of collagen and sarcoplasmic protein denaturation (Table S1, Supplemental data). Additionally, in general, there was no significant difference in shear force values and collagen solubilisation between these Stage 1 cooking treatments, even though there was a pattern of lower shear force values and higher solubilised collagen with cooking at 56 °C for 6 and 12 h compared to cooking at 46, 50, and 53 °C °C for 12 h (refer to Table 6.3 and Table 6.5 for Stage 1 only cooking). Furthermore, the shear force deformation curve shows a lower final yield value, which is associated with the low connective tissue strength of meat cooked at 56 °C for 6 and 12 h, compared to that at 46, 50, and 53 °C cooking for 12 h (Moller et al., 1981) (Figure S1, Supplemental data). Despite variations in cumulative cathepsin B and L activity, collagen denaturation degree, and final yield value during Stage 1 cooking, meat cooked using the 80_8 treatment at these Stage 1 cooking treatments exhibited similar shear force value. The lower shear force value in two-stage 80_8h treatment with stage 1 cooking at 46, 50, and 53 °C for 12 h

compared to one stage 80_8h treatment might suggest the contribution of cathepsin B and L in tenderisation. However, it is evident that tenderisation for two-stage 80_8h treatment occurs faster at Stage 1 cooking temperatures of 56 °C with low cumulative cathepsin B and L activity compared to at temperatures of 46, 50, and 53 °C, which is also in agreement with the results reported in the earlier chapter (Figure 4.14, Chapter 4). Taken together, although Stage 1 temperature and time can be used to manipulate cathepsin B and L activity, the efficiency of cathepsin B and L activity in tenderising meat for the two-stage cooking is relatively less compared to the tenderisation due to temperature and time effect on collagen denaturation and the weakening of collagen structure. The reduced ability of cathepsin B and L to tenderise meat during *sous vide* cooking may also be attributed to the increase in the pH value of meat from pH 5.5 (raw) to pH 5.9 with the increase in cooking temperature and time (Table 6.2). Several studies have reported that the activity of cathepsin B and L on myofibrillar proteins decreases as the pH increases from pH 5.5 to 6.0 (Lomiwes, Farouk, et al., 2014; Mikami et al., 1987). Furthermore, the degradation of collagen by cathepsin B and L is higher at a pH value of 3.5 – 4 compared to pH 6 (Kirschke et al., 1982; Shoji et al., 2014).

Although two-stage cooking improved meat tenderness compared to one-stage cooking for 80_8h treatment, no improvement in cooking loss and solubilised collagen was observed. The similar cooking loss between two-stage 80_8h treatment and one-stage 80_8h treatment suggests that Stage 1 cooking conditions might not affect factors contributing to the cooking loss in meat, such as protein oxidation and aggregation (Martens et al., 1982; Traore et al., 2012; Vaskoska, Ha, Ong, Chen, et al., 2021). Additionally, the comparable collagen solubilisation observed in meat subjected to two-stage 80_8h treatment and one-stage 80_8h treatment suggests that the difference in their shear force values might be attributed to variations in the mechanical strength of the residual collagen within the meat.

Overall, the one-stage 56_48h treatment produces the best results in terms of a low shear force value and minimising cooking loss. However, a two-stage process, which is shorter in total time, can potentially achieve the necessary tenderisation. It has been shown that two-stage 80_8h

treatment where the Stage 1 was performed at temperature 56 °C for 6 h and at 46, 50, 53, and 56 °C for 12 h has a lower shear force value compared to one-stage 80_8h treatment. Thus, to minimise the total processing time, it was decided that treatment at 56 °C for 6 h was selected as the Stage 1 treatment for the next experiment. The objective of the next experiment was to determine the effects of total cooking time and variations in the second stage temperature-time combinations on meat shear force value, cooking loss, and solubilised collagen.

6.3.2 Experiment 2 (Varied second stage and fixed first stage at 56 °C for 6h)

6.3.2.1 Beef brisket characteristic

Table 6. 6 Characteristic of raw and *sous vide* cooked beef brisket at 71 °C (T_C).

Attribute	Mean ± SEM
Raw brisket	
pH	5.53 ± 0.02
Total collagen (mg g^{-1} meat)	8.0 ± 0.3
***Sous vide* cooked brisket at 71 °C**	
Peak shear force (N)	69.6 ± 2.7
Cooking loss (% w/w)	29.4 ± 0.5

Tc (the core temperature of meat reaches the applied temperature).
n = 8 animal replicates.

Table 6.6 showed the properties of raw meat and meat *sous vide* cooked at 71 °C (Tc). The pH value of raw meat used in this study is in the range of normal post-rigor pH value of beef. The value cooking loss was significantly higher compared to the value reported in Experiment 1 of this chapter (P = 0.0153). This could be due to the different in aging time (11 vs. 8 days) used in this study. The higher cooking loss with extended aging has been reported in several studies (Purslow et al., 2016; Vaskoska et al., 2020).

6.3.2.2 Peak shear force

Table 6.7 shows the shear force value of beef brisket *sous vide* cooked using different cooking treatments (the data was illustrated in Figure 6.6). There was a two-way interaction between temperature and time, as well as between temperature and cooking procedure (conventional vs. two-stage) on shear force value of meat. In general, the increase in cooking time resulted in a

decrease in shear force values. Additionally, there was a trend of lower shear force values of meat cooked using a two-stage compared to conventional cooking, particularly at cooking temperatures of 70, 80, and 90 °C. However, a significantly lower shear force value was only observed at a temperature of 70 °C for cooking times of 2 and 20 h. The increase in shear force value of meat with the increase in cooking temperature from 60 to 70 °C has been reported in various studies (Beilken et al., 1986; Christensen, Purslow, & Larsen, 2000; Vaskoska et al., 2020; Vaskoska, Ha, Ong, Chen, et al., 2021). It has been reported that cooking above 60 – 65 °C resulted in actin denaturation and the aggregation of myofibrillar proteins, which is related to the longitudinal shrinkage of meat and a decrease in sarcomere length (Martens et al., 1982; Palka & Daun, 1999; Promeyrat, Bax, et al., 2010; Vaskoska, Ha, Ong, Chen, et al., 2021; Zielbauer et al., 2016). Subsequently, there was a greater overlap of actin and myosin, along with an increase in the amount of connective tissue per unit sarcomere length (Bouton, Harris, Shorthose, & Ratcliff, 1974). Thus, the reduction in shear force value in the two-stage cooking compared to conventional cooking, which was observed primarily at 70 °C cooking treatments, could be due to the weakening of connective tissue during the Stage 1 treatment. This subsequently reduced the effective contribution of connective tissue per unit sarcomere length when the meat was further cooked at 70 °C (Bouton et al., 1974). As the second stage cooking temperature increases, the impact of Stage 1 cooking becomes negligible. This might be due to the weakening of connective tissue was also promoted at a higher cooking treatment due to a greater collagen solubilisation (Table 6.9) and collagen degradation (Christensen et al., 2000; Wang et al., 2022). Additionally, when considering the total cooking time, there were no significant differences in the shear force values of meat between conventional and two-stage cooking.

Table 6. 7 Peak shear force value (N) (means ± SEM) of beef brisket *sous vide* cooked using two cooking procedure (conventional and two-stage cooking). Conventional *sous vide* cooking involves cooking at 60, 70, 80, and 90 °C for different time periods (2, 6, 8, 12, 14, 20, and 26 h). Two-stage *sous vide* cooking involves first stage cooking at 56 °C for 6 h and second stage at 60, 70, 80, and 90 °C for different time periods (0, 2, 6, 8, 14, and 20 h). Different lowercase letters (a-e) within the same column and capital letter (A-B) within the same row statistically indicate the significant differences ($p < 0.05$).

Sous vide	Time	Temperature (°C)			
	(h)	**60**	**70**	**80**	**90**
Conventional	2	$63.4 \pm 5.4_{ab}{}^{B}$	$95.1 \pm 2.1_{a}{}^{A}$	$63.0 \pm 2.4_{ab}{}^{B}$	$57.3 \pm 4.0_{ab}{}^{B}$
	6	$61.9 \pm 3.5_{abc}{}^{AB}$	$78.6 \pm 0.8_{ab}{}^{A}$	$57.1 \pm 5.3_{abc}{}^{B}$	$53.0 \pm 3.2_{abc}{}^{B}$
	8	$69.8 \pm 6.0_{a}{}^{A}$	$66.7 \pm 1.9_{bc}{}^{AB}$	$57.2 \pm 3.1_{abcd}{}^{BC}$	$48.6 \pm 4.9_{bc}{}^{C}$
	12	–	–	$49.3 \pm 4.6_{cde}{}^{A}$	$42.8 \pm 0.4_{bc}{}^{A}$
	14	$57.0 \pm 1.4_{abcd}{}^{A}$	$66.0 \pm 4.4_{bcd}{}^{A}$	$42.9 \pm 0.9_{de}{}^{B}$	$44.9 \pm 2.5_{bc}{}^{B}$
	20	$49.2 \pm 1.5_{bcd}{}^{AB}$	$62.1 \pm 0.9_{bcd}{}^{A}$	$45.2 \pm 4.8_{cde}{}^{B}$	$41.4 \pm 3.3_{c}{}^{B}$
	26	$44.6 \pm 1.6_{d}{}^{A}$	$46.5 \pm 2.8_{ef}{}^{A}$	–	–
Two-stage	0	$56.7 \pm 5.4_{abcd}{}^{A}$	$65.5 \pm 4.4_{bcd}{}^{A}$	$68.4 \pm 2.6_{a}{}^{A}$	$66.1 \pm 4.2_{a}{}^{A}$
	2	$61.4 \pm 6.3_{abc}{}^{A}$	$60.3 \pm 4.9_{cde}{}^{A}$	$55.4 \pm 2.1_{abcd}{}^{A}$	$49.9 \pm 0.9_{bc}{}^{A}$
	6	–	–	$49.9 \pm 1.3_{bcd}{}^{A}$	$41.3 \pm 1.9_{c}{}^{B}$
	8	$56.6 \pm 3.6_{abc}{}^{A}$	$56.4 \pm 2.6_{cdef}{}^{A}$	$42.8 \pm 2.1_{cde}{}^{B}$	$39.6 \pm 2.8_{c}{}^{B}$
	14	$52.6 \pm 4.3_{bcd}{}^{A}$	$51.3 \pm 1.8_{def}{}^{A}$	$39.7 \pm 4.1_{e}{}^{B}$	$46.1 \pm 2.5_{bc}{}^{AB}$
	20	$50.7 \pm 2.2_{cd}{}^{A}$	$47.3 \pm 4.6_{f}{}^{A}$	–	–
P value					
Temp		<0.0001			
Time		<0.0001			
CP		<0.0001			
Temp x time		0.0003			
Temp x CP		0.0034			
Temp x time x CP		0.2947			

Abbreviations: Temp (Temperature) and CP (coking procedure, conventional vs. two-stage)

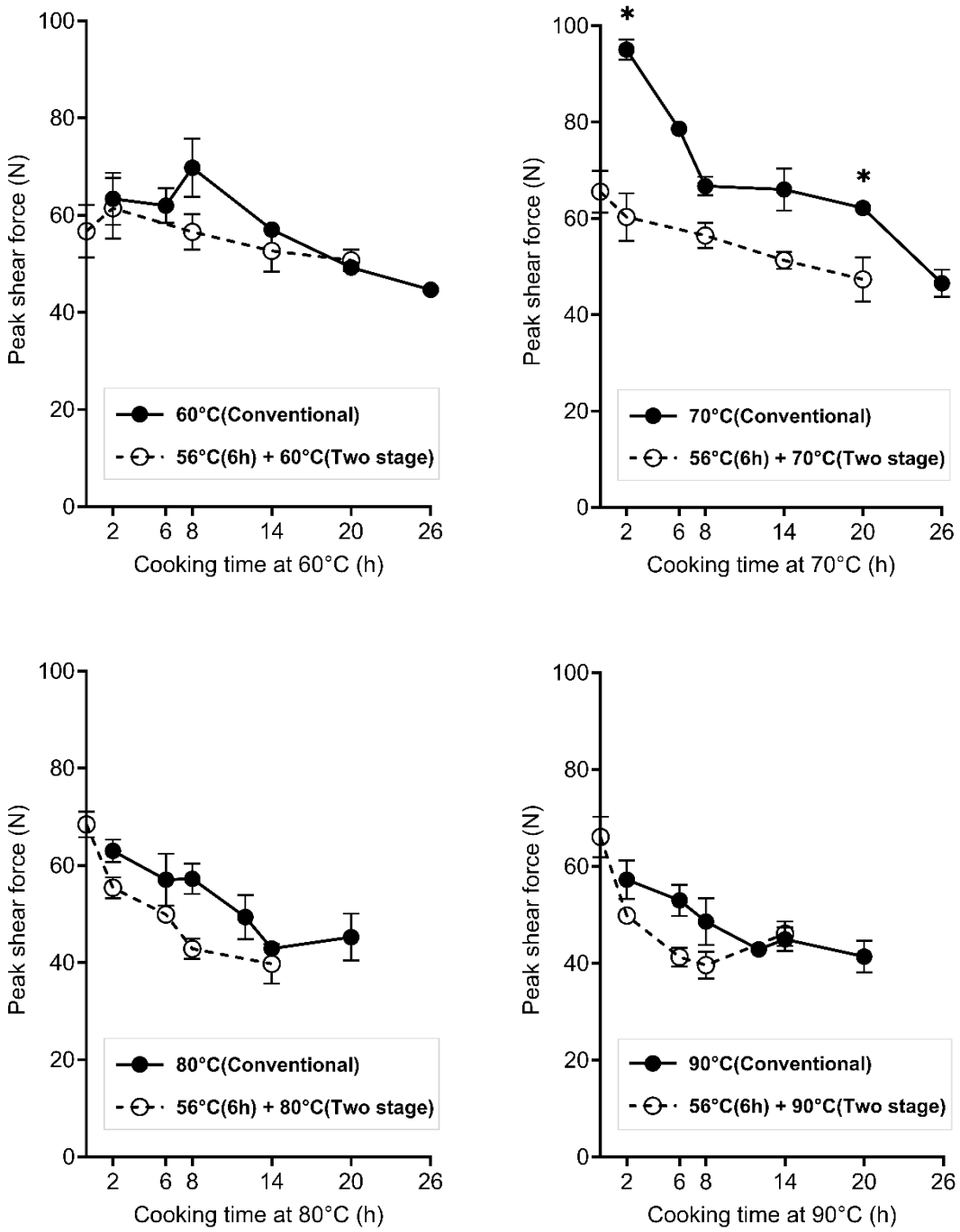

Figure 6. 6 Representation of result described in Table 6.7. Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). The asterisk (*) and asterisk above bar ($\overset{*}{\vdash\!\dashv}$) indicate the significant differences ($p < 0.05$) between conventional and two-stage *sous vide* cooking within the same Stage 2 cooking time and total cooking time, respectively.

6.3.2.3 Cooking loss

Table 6. 8 Cooking loss (%) (means ± SEM) of beef brisket *sous vide* cooked using two cooking procedure (conventional and two-stage cooking). Conventional *sous vide* cooking involves cooking at 60, 70, 80, and 90 °C for different time periods (2, 6, 8, 12, 14, 20, and 26 h). Two-stage *sous vide* cooking involves first stage cooking at 56 °C for 6 h and second stage at 60, 70, 80, and 90 °C for different time periods (0, 2, 6, 8, 14, and 20 h). Different lowercase letters (a-e) within the same column and capital letter (A-B) within the same row statistically indicate the significant differences ($p < 0.05$).

Sous vide	Time	Temperature (°C)			
	(h)	**60**	**70**	**80**	**90**
Conventional	2	$29.2 \pm 0.8_{abc}{}^{A}$	$39.3 \pm 0.8_{bc}{}^{B}$	$43.1 \pm 0.4_{ab}{}^{C}$	$45.2 \pm 0.9_{ab}{}^{D}$
	6	$29.4 \pm 0.9_{abcd}{}^{A}$	$40.7 \pm 1.3_{cde}{}^{B}$	$43.1 \pm 1.4_{b}{}^{BC}$	$44.6 \pm 0.9_{ab}{}^{C}$
	8	$31.4 \pm 1.6_{cde}{}^{A}$	$41.2 \pm 0.9_{cde}{}^{B}$	$42.8 \pm 0.6_{b}{}^{B}$	$45.6 \pm 1.0_{ab}{}^{B}$
	12	–	–	$44.0 \pm 0.7_{b}{}^{A}$	$43.8 \pm 0.3_{ab}{}^{A}$
	14	$31.5 \pm 1.2_{de}{}^{A}$	$41.7 \pm 1.0_{cde}{}^{B}$	$42.9 \pm 0.8_{b}{}^{B}$	$43.5 \pm 0.5_{ab}{}^{B}$
	20	$33.8 \pm 0.8_{e}{}^{A}$	$42.4 \pm 0.9_{e}{}^{B}$	$44.9 \pm 0.8_{b}{}^{B}$	$45.7 \pm 0.6_{ab}{}^{B}$
	26	$34.0 \pm 1.5_{e}{}^{A}$	$43.6 \pm 1.1_{de}{}^{B}$	–	–
Two-stage	0	$25.9 \pm 1.8_{a}{}^{A}$	$32.6 \pm 1.2_{a}{}^{B}$	$38.4 \pm 1.6_{a}{}^{C}$	$41.6 \pm 1.2_{a}{}^{D}$
	2	$27.0 \pm 0.6_{ab}{}^{A}$	$35.1 \pm 1.5_{ab}{}^{B}$	$43.7 \pm 0.7_{ab}{}^{C}$	$45.7 \pm 0.2_{ab}{}^{D}$
	6	–	–	$43.3 \pm 0.8_{b}{}^{A}$	$44.9 \pm 0.9_{ab}{}^{A}$
	8	$26.7 \pm 0.7_{abc}{}^{A}$	$38.7 \pm 1.6_{bcd}{}^{B}$	$44.5 \pm 1.4_{b}{}^{C}$	$46.4 \pm 0.9_{b}{}^{C}$
	14	$30.5 \pm 1.5_{bcde}{}^{A}$	$41.4 \pm 0.8_{cde}{}^{B}$	$43.7 \pm 1.5_{b}{}^{B}$	$47.8 \pm 0.4_{b}{}^{C}$
	20	$34.7 \pm 1.2_{e}{}^{A}$	$40.2 \pm 1.5_{cde}{}^{B}$	–	–
P value					
Temp		<0.0001			
Time		<0.0001			
CP		0.2742			
Temp x time		0.0047			
Temp x CP		0.0001			
Temp x time x CP		0.1902			

Abbreviations: Temp (Temperature) and CP (coking procedure, conventional vs. two-stage)

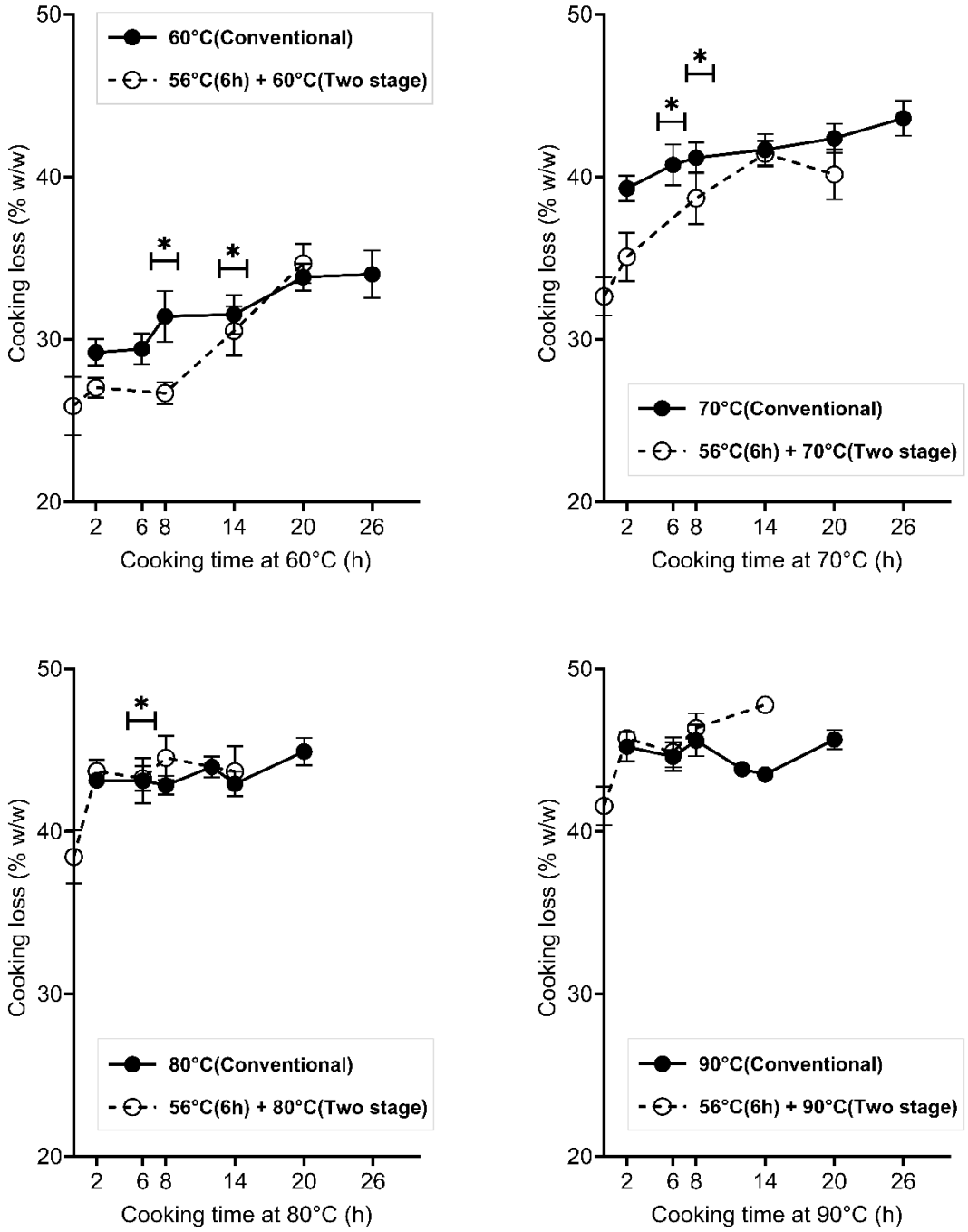

Figure 6. 7 Representation of result described in Table 6.8. Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). The asterisk (*) and asterisk above bar ($\overset{*}{\vdash\dashv}$) indicate the significant differences ($p < 0.05$) between conventional and two-stage *sous vide* cooking within the same cooking time and total cooking time, respectively.

6.3.2.4 Solubilised collagen in cook loss

Table 6. 9 Solubilised collagen in cook loss per total collagen (%) (means ± SEM) of beef brisket *sous vide* cooked using two cooking procedure (conventional and two-stage cooking). Conventional *sous vide* cooking involves cooking at 60, 70, 80, and 90 °C for different time periods (2, 6, 8, 12, 14, 20, and 26 h). Two-stage *sous vide* cooking involves first stage cooking at 56 °C for 6 h and second stage at 60, 70, 80, and 90 °C for different time periods (0, 2, 6, 8, 14, and 20 h). Different lowercase letters (a-e) within the same column and capital letter (A-B) within the same row statistically indicate the significant differences ($p < 0.05$).

Sous vide	Time	Temperature (°C)			
	(h)	**60**	**70**	**80**	**90**
Conventional	2	$3.5 \pm 0.2_{ab}{}^{A}$	$6.0 \pm 1.5_{ab}{}^{B}$	$6.7 \pm 0.9_{ab}{}^{B}$	$8.8 \pm 0.5_{ab}{}^{C}$
	6	$3.8 \pm 0.2_{ab}{}^{A}$	$6.7 \pm 1.6_{abc}{}^{B}$	$10.7 \pm 3.0_{c}{}^{C}$	$16.5 \pm 2.7_{c}{}^{D}$
	8	$4.3 \pm 0.8_{abc}{}^{A}$	$6.1 \pm 0.8_{abc}{}^{B}$	$10.6 \pm 1.3_{cd}{}^{C}$	$25.8 \pm 2.4_{cde}{}^{D}$
	12	–	–	$10.4 \pm 0.9_{cd}{}^{A}$	$27.2 \pm 2.6_{def}{}^{B}$
	14	$4.5 \pm 0.5_{abc}{}^{A}$	$9.1 \pm 1.2_{bc}{}^{B}$	$15.4 \pm 1.2_{d}{}^{C}$	$28.8 \pm 2.2_{ef}{}^{D}$
	20	$5.1 \pm 0.5_{abc}{}^{A}$	$10.3 \pm 1.5_{cd}{}^{B}$	$21.5 \pm 1.9_{e}{}^{C}$	$40.5 \pm 3.3_{g}{}^{D}$
	26	$6.1 \pm 1.2_{bc}{}^{A}$	$15.1 \pm 2.4_{d}{}^{B}$	–	–
Two-stage	0	$3.2 \pm 0.6_{a}{}^{A}$	$5.4 \pm 0.8_{a}{}^{B}$	$5.5 \pm 0.6_{a}{}^{B}$	$6.3 \pm 0.6_{a}{}^{B}$
	2	$3.9 \pm 0.3_{ab}{}^{A}$	$4.7 \pm 1.3_{aA}{}^{B}$	$8.7 \pm 0.1_{abc}{}^{BC}$	$9.8 \pm 1.1_{b}{}^{C}$
	6	–	–	$9.1 \pm 0.5_{bc}{}^{A}$	$17.4 \pm 0.6_{c}{}^{B}$
	8	$3.4 \pm 0.1_{ab}{}^{A}$	$6.6 \pm 0.5_{abc}{}^{B}$	$11.4 \pm 0.3_{cd}{}^{C}$	$21.1 \pm 2.0_{cd}{}^{D}$
	14	$4.3 \pm 0.3_{abc}{}^{A}$	$8.3 \pm 0.4_{bcd}{}^{B}$	$15.5 \pm 1.6_{d}{}^{C}$	$33.8 \pm 3.3_{fg}{}^{D}$
	20	$7.2 \pm 0.5_{c}{}^{A}$	$10.0 \pm 1.5_{cd}{}^{B}$	–	–
P value					
Temp		<0.0001			
Time		<0.0001			
CP		0.0924			
Temp x time		<0.0001			
Temp x time x CP		0.6701			

Abbreviations: Temp (Temperature) and CP (coking procedure, conventional vs. two-stage)

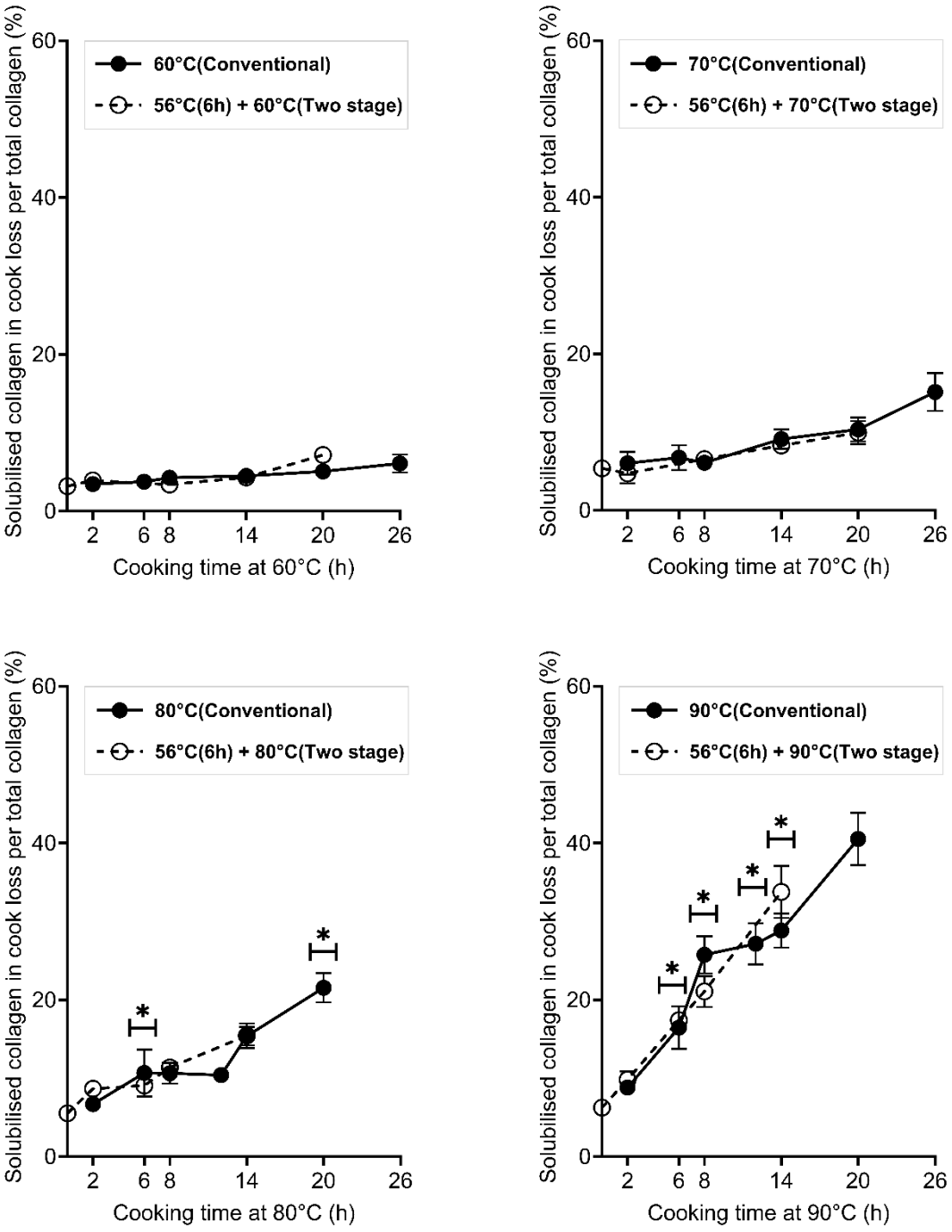

Figure 6. 8 Representation of result described in Table 6.9. Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). The asterisk (*) and asterisk above bar ($\overset{*}{\vdash\!\dashv}$) indicate the significant differences ($p < 0.05$) between conventional and two-stage *sous vide* cooking within the same cooking time and total cooking time, respectively.

Table 6.8 shows the cooking loss of beef brisket *sous vide* cooked using different cooking treatments (the data was illustrated in Figure 6.7). There was a significant interaction between temperature and time, as well as between temperature and cooking procedure (conventional vs. two-stage), affecting the cooking loss of meat. The increase in cooking time led to a higher cooking loss, particularly at temperatures of 60 and 70 °C. Additionally, there was a trend of lower cooking loss in two-stage cooking, particularly at a cooking temperature of 60 °C (2 and 8 h) and 70 °C (2 h). This trend of lower cooking loss in two-stage cooking, compared to conventional cooking, may be attributed to the improvement of the water-holding capacity of meat due to the denaturation of sarcoplasmic proteins during the Stage 1 cooking, forming a three-dimensional network that traps water (Figure 5.4, Chapter 5). Furthermore, the formation of this protein network in the extracellular space might reduce the porosity of meat. These changes, altogether, could reduce the migration of water to the surface of the meat caused by actin denaturation, myofibrillar aggregation, and longitudinal shrinkage when the meat is subsequently cooked at the second-stage temperatures (Feyissa et al., 2013; Liu, Arner, Puolanne, & Ertbjerg, 2016). This is in contrast to meat that is directly cooked at second-stage temperatures, where the denaturation and aggregation of different meat proteins occurs in parallel. However, at cooking temperatures of 80 and 90 °C, and prolonged cooking time at 60 and 70 °C, the cooking loss between two-stage and conventional cooking becomes comparable. This observation could be attributed to the greater aggregation of sarcoplasmic protein, which was favoured at higher cooking treatments, leading to the release of water from the gel network system (Zhao, Zhang, Zhao, & Xu, 2023).

Interestingly, when considering the total cooking time, there were significant differences in the cooking loss of meat between conventional and two-stage cooking, particularly at temperatures 60, 70, and 80 °C, at short cooking times. For example, meat cooked using two-stage cooking at 60 °C for 2 h and 8 h, 70 °C for 0 h and 2 h, and 80 °C for 0 h exhibited significantly lower cooking loss compared to their conventional counterparts under similar total cooking times. Similarly, Ismail et al. (2019) reported a lower cooking loss in meat cooked using two-stage cooking compared conventional cooking under similar total cooking time. The lower cooking loss

observed in two-stage cooking may simply be attributed to the shorter cooking time spent at high temperatures in two-stage cooking compared to conventional cooking when evaluated under similar total cooking time.

When comparing the results of cooking loss and shear force values, meat cooked using two-stage cooking at 60 °C for 2 h and 8 h, 70 °C for 0 h and 2 h, and 80 °C for 0 h was not significantly different in shear force values but had lower cooking loss compared to its conventional counterparts under similar total cooking time. This suggests the benefit of two-stage cooking over conventional cooking in reducing cooking loss at these temperature treatments under a similar total cooking time. However, it is worth noting that in these temperature and time treatments, the meat still has a high shear force value in the range of 56.6 – 68.4 N. Therefore, these temperature and time are not sufficient to achieve tenderisation.

Table 6.9 shows the solubilised collagen in beef brisket *sous vide* cooked using different cooking treatments (the data was illustrated in Figure 6.8). There was a significant interaction between temperature and time on solubilised collagen. The increase in cooking time resulted in an increase in solubilised collagen especially at temperature 70, 80, and 90 °C. Furthermore, there was a significant difference in the solubilised collagen of meat cooked using a two-stage cooking method compared to conventional cooking when evaluated under similar total cooking times, particularly at temperatures 80 and 90 °C. For example, meat that was cooked using conventional methods at 80 °C for 6 h and 20 h, and 90 °C for 6, 8, 14, and 20 h, exhibited significantly higher solubilised collagen compared to their two-stage counterparts under similar total cooking times. This result suggests that Stage 1 treatment did not promote collagen solubilisation, and the variation in collagen solubilisation was mainly affected by Stage 2 treatment, aligning with the findings obtained in Experiment 1. Additionally, this result implies that the significantly lower shear force value in meat cooked using the two-stage cooking compared to conventional cooking at 70°C for 2 and 20 h, might be attributed to factors other than collagen solubilisation, such as differences in collagen mechanical strength attributed to Stage 1 cooking.

6.3.2.5 Conventional one-stage *sous vide* cooking

Table 6. 10 Peak shear force value (N), cooking loss (%), solubilised collagen in cook loss per total collagen, SC (%) (means ± SEM) of beef brisket *sous vide* cooked using conventional one-stage *sous vide* cooking at 60, 70, 80, and 90 °C for different time periods (2, 6, 8, 12, 14, 20, and 26 h). Different lowercase letters (a-e) within the same column statistically indicate the significant differences ($p < 0.05$).

Temperature (°C)	Time (h)	Peak shear force (N)	Cooking loss (%)	SC (%)
60	2	63.4 ± 5.4efg	29.2 ± 0.8a	3.5 ± 0.2a
	6	61.9 ± 3.5defg	29.4 ± 0.9a	3.8 ± 0.2a
	8	69.8 ± 6.0fg	31.4 ± 1.6ab	4.3 ± 0.8ab
	14	57.0 ± 1.4abcdef	31.5 ± 1.2ab	4.5 ± 0.5abc
	20	49.2 ± 1.5abcdef	33.8 ± 0.8bc	5.1 ± 0.5abc
	26	44.6 ± 1.6abc	34.0 ± 1.5bc	6.1 ± 1.2abc
70	2	95.1 ± 2.1h	39.3 ± 0.8cd	6.0 ± 1.5abc
	6	78.6 ± 0.8gh	40.7 ± 1.3de	6.7 ± 1.6bcd
	8	66.7 ± 1.9fg	41.2 ± 0.9def	6.1 ± 0.8bcd
	14	66.0 ± 4.4efg	41.7 ± 1.0def	9.1 ± 1.2cde
	20	62.1 ± 0.9cdefg	42.4 ± 0.9def	10.3 ± 1.5def
	26	46.5 ± 2.8abc	43.6 ± 1.1def	15.1 ± 2.4efg
80	2	63.0 ± 2.4efg	43.1 ± 0.4def	6.7 ± 0.9abc
	6	57.1 ± 5.3bcdef	43.1 ± 1.4ef	10.7 ± 3.0def
	8	57.2 ± 3.1abcdef	42.8 ± 0.6ef	10.6 ± 1.3def
	12	49.3 ± 4.6abcde	44.0 ± 0.7ef	10.4 ± 0.9defg
	14	42.9 ± 0.9ab	42.9 ± 0.8ef	15.4 ± 1.2fg
	20	45.2 ± 4.8abc	44.9 ± 0.8f	21.5 ± 1.9hi
90	2	57.3 ± 4.0abcdef	45.2 ± 0.9ef	8.8 ± 0.5cde
	6	53.0 ± 3.2abcdef	44.6 ± 0.9ef	16.5 ± 2.7gh
	8	48.6 ± 4.9abcd	45.6 ± 1.0ef	25.8 ± 2.4hi
	12	42.8 ± 0.4abc	43.8 ± 0.3ef	27.2 ± 2.6hi
	14	44.9 ± 2.5abc	43.5 ± 0.5ef	28.8 ± 2.2i
	20	41.4 ± 3.3a	45.7 ± 0.6f	40.5 ± 3.3j

When considering the total cooking time, adding the Stage 1 cooking step to the *sous vide* cooking process did not provide an additional benefit in terms of reducing the shear force value compared to cooking using conventional one-stage *sous vide* cooking. Therefore, the next discussion will focus on conventional one-stage *sous vide* cooking. In order to better illustrate the effect of temperature and time on shear force value, cooking loss, and solubilised collagen of beef brisket cooked using conventional *sous vide* cooking, Table 6.10 and Figure 6.9 was constructed using

the data From Table 6.7, 6.8, and 6.9 for shear force value, cooking loss, and solubilised collagen, respectively.

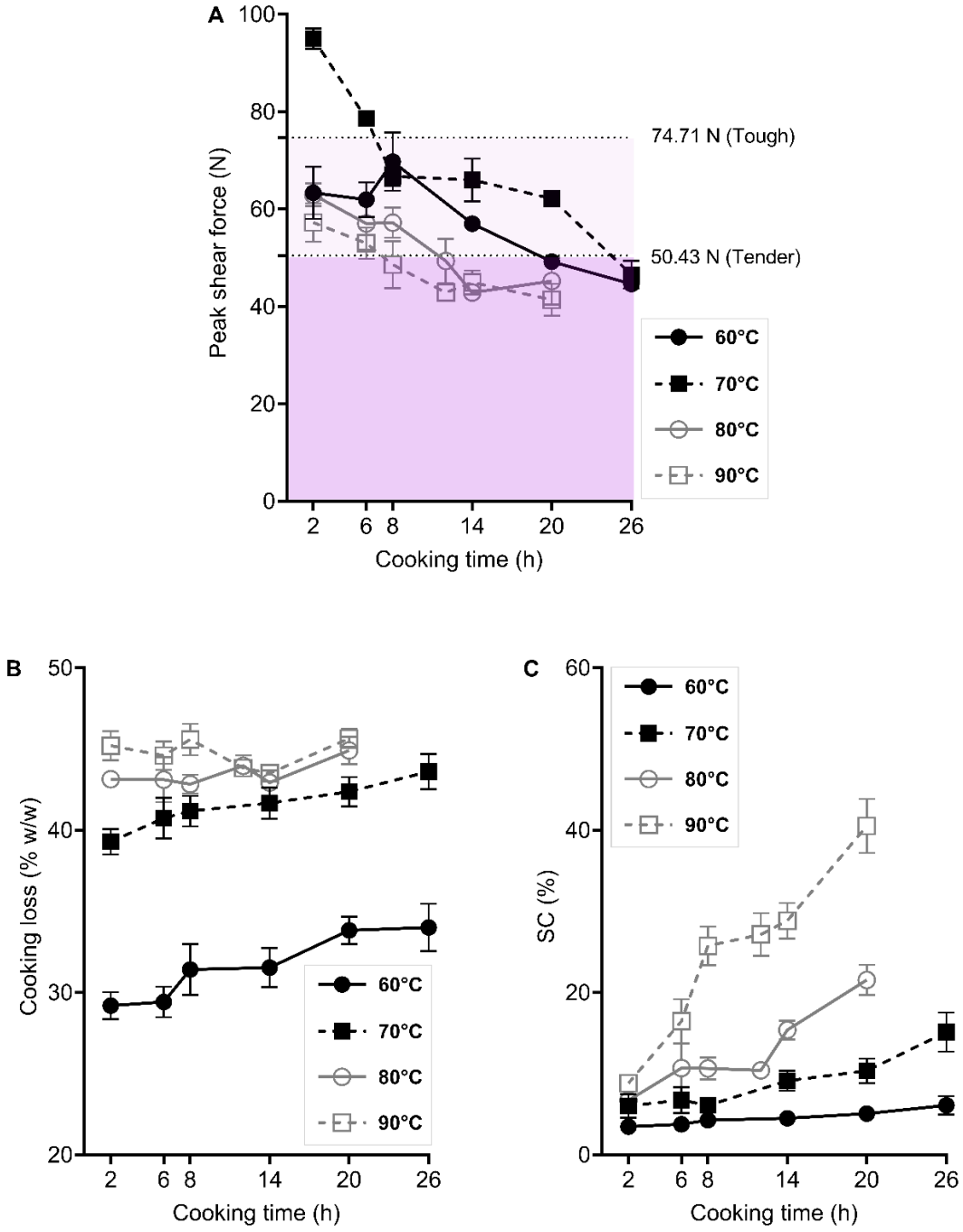

Figure 6. 9 Representation of result described in Table 6.10. A. Peak shear force value (N), B. Cooking loss (%), and C. Solubilised collagen in cook loss per total collagen (%). Vertical bars represent SEM (for some means error bars are shorter than the size of the symbol). Dashed line in Figure A represents consumer threshold for tough (74.71 N) and tender (50.43 N) meat.

Based on the regression relationship between consumer tenderness ratings and shear force values, Liang et al. (2016) classified meat with a shear force value of >55.43 N as tough, 41.4 – 55.43 N as intermediate, and <41.4 N as tender. In their study, they utilised a similar instrument and blade as those employed in this study but with different sample dimensions. In the present study, a square cross-section sample (1.0 cm × 1.0 cm) was used, while Liang et al. (2016) employed a round cross-section (1.27 cm in diameter). To convert the tenderness threshold values obtained in their study to the equivalent shear force values in this study, a conversion factor reported by Silva et al. (2015) was applied. This yielded tenderness threshold values of >74.71 N (tough), 50.43 – 74.71 N (intermediate), and <50.43 N (tender), as illustrated in Figure 6.9 A. A conversion method to equate shear force value when different shear force measurement settings were used has also been reported by Rosenvold et al. (2008).

It can be seen from Figure 6.9A that cooking at 60, 80, and 90 °C for just 2 h already resulted in meat with shear force values below the toughness threshold of 74.71 N. However, at 70 °C, prolonged cooking for 8 h was needed to reduce the shear force value below the same threshold. Additionally, the time needed to achieve consumer tenderness acceptability, defined as a shear force value below 50.43 N, varies based on cooking temperature. At 60 °C, it took 20 h to reduce the shear force value below the tenderness threshold. In contrast, shorter cooking times of 12 h and 8 h were required at 80 °C and 90 °C, respectively. Furthermore, cooking at 70 °C required the longest cooking time which was 26 h to reach the tenderness threshold compared to other cooking temperatures. These cooking treatments resulted in meat with no significant difference in shear force values, which were 49.2, 46.5, 49.3, and 48.6 N for 60, 70, 80, and 90 °C cooking treatments, respectively (Table 6.10).

In terms of cooking loss, cooking at 60 °C resulted in a lower cooking loss compared to other cooking temperatures. Increasing the cooking temperature from 60 to 70 °C caused a marked increase in cooking loss (Table 6.10). Similarly, Zielbauer et al. (2016) and Supaphon et al. (2021) reported a significant increase in cooking loss with an increase in cooking temperature from 60 to 70 °C. This marked increase was associated with collagen (shrinkage of connective tissue) and

actin denaturation (Brunton, Lyng, Zhang, & Jacquier, 2006; Ishiwatari, Fukuoka, & Sakai, 2013; Tornberg, 2005; Zielbauer et al., 2016). However, cooking at 60 °C for 2 h already produces complete collagen denaturation (Figure 5.4, Chapter 5). Furthermore, Zielbauer et al. (2016) reported that complete actin denaturation was achieved with cooking at 60 °C for 24 h, as evidenced by the complete disappearance of meat's endothermic curve obtained by differential scanning calorimetry (DSC). Thus, this suggests that collagen and actin denaturation could contribute to the cooking loss in the range of 29.2 – 34.0 % during prolonged cooking at a temperature of 60 °C. While the marked increase from 60 to 70 °C could be attributed to other factors, such as subsequent oxidation and aggregation of myofibrillar proteins, which might cause a reduction in meat water holding capacity, as discussed in Section 6.3.1 (Estévez, 2011; Martens et al., 1982; Promeyrat et al., 2013; Promeyrat, Gatellier, et al., 2010; Traore et al., 2012; Xu et al., 2023).

The decrease in the peak shear force value with an increase in cooking time might be attributed to the increase in the amount of solubilised collagen, as depicted in Figure 6.9C. This correlation is further supported by a significant moderate correlation between solubilised collagen and shear force value ($r = –0.54$, $p < 0.0001$) (Figure 6.10). However, it was evident that the amount of solubilised collagen required to cause a reduction in shear force value below the tenderness level (<50.43 N) is dependent on temperature. At 60 °C, the increase in solubilised collagen to 5.1 % was sufficient to reduce the shear force value below the tenderness threshold. On the other hand, at 70, 80, and 90 °C, significantly higher amounts of solubilised collagen were required (10.6 – 25.8 %) (Table 6.10). This could be associated with greater longitudinal shrinkage at these temperatures compared to 60 °C, causing an increase in the amount of connective tissue per unit sarcomere length, as discussed in Section 6.3.2.2 (Bouton et al., 1974). Thus, in order to reduce the effective contribution of connective tissue per unit sarcomere length, a greater reduction in connective tissue strength was required through greater collagen degradation and solubilisation.

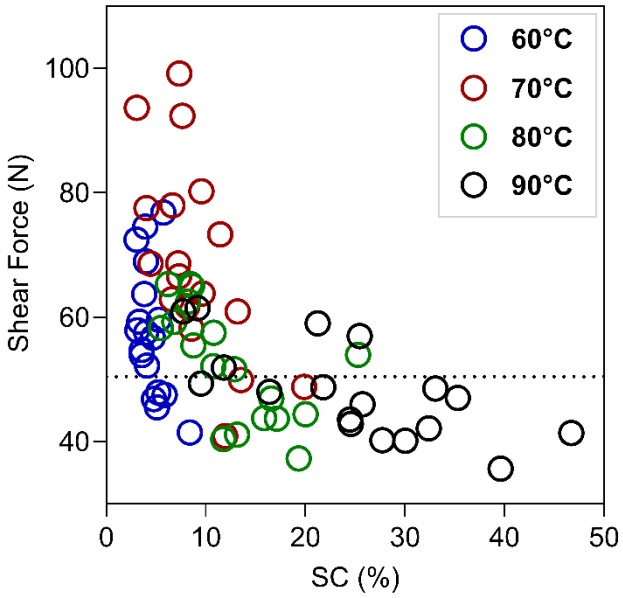

Figure 6. 10 Scatter plots of shear force value vs. solubilised collagen in cook loss per total collagen (SC) of beef brisket *sous vide* cooked at different temperature and time combinations. N=72. Dashed line represents consumer threshold for tender meat (50.43 N).

6.4 Conclusion

The two-stage 80_8h cooking treatment, with Stage 1 involving cooking at 46, 50, and 53 °C for 12 h, and at 56 °C for 6 and 12 h, resulted in a lower shear force value compared to conventional one-stage cooking at 80 °C for 8 h. Subsequently, when using a greater variation in temperature-time combinations for Stage 2 and 56 °C for 6 h as the Stage 1 treatment, no benefit of two-stage cooking over conventional one-stage cooking on shear force values was observed, especially when considering total cooking time. On the other hand, two-stage cooking can offer benefits in terms of a reduction in cooking loss. However, this benefit was only observed at certain temperature and time combinations, particularly at a shorter cooking time, where the shear force value remained high in the range of 56.6 – 68.4 N.

Based on conventional one-stage cooking, at 60 °C, it took 20 h to reduce the shear force value below the tenderness threshold, but it has an advantage in terms of low cooking loss (33.8 %). Cooking at higher temperatures of 80 °C and 90 °C reduces the time to 12 and 8 h to reach the tenderness threshold, but at the expense of high cooking loss with 44.0 % and 45.6 %, respectively. Furthermore, cooking beef brisket at 70 °C is the least favourable option. This temperature requires the longest cooking time (26 h) to reach the tenderness threshold compared to other cooking temperatures and resulted in a high cooking loss of 43.6 %, similar to 80 °C and 90 °C.

CHAPTER 7 Final discussion and future work

7.1 Final discussion

Sous vide cooking has been shown to tenderise tough meat cuts (Baldwin, 2012; Christensen et al., 2011; Fareh, 2018; Gámbaro et al., 2023; Naqvi et al., 2021). However, this process requires a long cooking time, which is not feasible to be applied in the food industry. Using a two-stage *sous vide* cooking may provide a solution to reduce the total cooking time compared to conventional single-temperature *sous vide* cooking. It is hypothesised that two-stage *sous vide* cooking can act on two mechanisms: meat protein proteolysis by endogenous enzymes, such as cathepsins B and L, during the low-temperature *sous vide* cooking (first stage) and rapid collagen solubilisation during the high-temperature *sous vide* cooking (second stage). Since tenderness is an important characteristic that determines consumer eating satisfaction, improving the tenderness of tough meat cuts like brisket can increase their value, thus benefiting the meat industry (Warner et al., 2022).

The involvement of cathepsin B and L in meat tenderness has been disputed since their localisation inside lysosomes prevents the ability of these enzymes to access substrates (Koohmaraie & Geesink, 2006). However, heat treatment has been shown to cause the release of cathepsin B and L from lysosomes, as evidenced by the increase in their activity in the sarcoplasmic fraction after heat treatment (Ertbjerg et al., 2012; Kaur et al., 2020). The released cathepsins may have the potential to come into contact with meat proteins and contribute to their degradation, subsequently improving meat tenderness. In agreement with previous studies, this thesis (Chapter 3) found that *sous vide* cooking beef brisket at temperatures of 46 to 60 °C resulted in an increase in cathepsin B and L activity to about four times of their activity in raw meat, before decreasing with further cooking time. Since enzymes are proteins, the decrease is attributed to heat-induced enzyme denaturation. These changes in cathepsin B and L activity during *sous vide* cooking have been widely reported (Christensen et al., 2013; Dominguez-Hernandez & Ertbjerg, 2021; Ertbjerg et al., 2012; Kaur et al., 2020). However, no study has been done to model their

kinetic activity. In this thesis, two kinetic models to predict cathepsin B and L activity in intact meat during *sous vide* cooking were developed based on consecutive reactions in series, which are activation (release of cathepsin from the lysosome) followed by deactivation (thermal denaturation). The first model assumed that the kinetics of cathepsin B and L activation and deactivation followed a first-order reaction and that the enzymes exhibited a single thermal stability. A good prediction was obtained with R^2=0.900. The results show that the deactivation reaction is more temperature-dependent than the activation reaction. From the model, maximum cathepsin B and L activity can be achieved in less than 1 h at cooking temperatures between 46 and 60 °C, with faster activation at higher cooking temperatures (52 min at 46 °C and 30 sec at 60 °C). The second model assumed the presence of two isoenzymes with different thermal stabilities. This is justifiable since the substrate used can be catalysed by both cathepsin B and cathepsin L. The model shows a slight improvement compared to the first model, with R^2=0.908. However, since the first model has fewer parameters and an acceptable R^2, the first model was used for the subsequent analysis. In this thesis, the effect of heat treatment on cathepsin B and L rate of reaction was also determined which shows an activation energy (Ea) of 35 kJ.mol^{-1} ± 2.87. The first model and the activation energy were combined to calculate cumulative cathepsin B and L activity during *sous vide* cooking. The results show that a greater cumulative activity can be achieved with *sous vide* cooking at lower temperatures such as 46 °C compared to higher temperatures such as 56 °C. Since heat treatment effect both cathepsin B and L activity and their rate of reaction, the use of cumulative activity can provide a better way to understand the relationship between cathepsins B and L and meat tenderness than the commonly used cathepsin B and L activity/ residual activity.

Since cathepsins B and L can degrade myofibrillar and connective tissue proteins, it is hypothesised that cooking at temperatures promoting greater cumulative activity could result in improved meat tenderness. To answer this, Chapter 4 examined the effects of precooking at 46 °C and 56 °C for up to 72 h, representing low and high cumulative activities, respectively, before cooking at 71 °C on meat tenderness, cooking loss, and collagen solubilisation. A good agreement

was obtained when the kinetics model in Chapter 3 was compared with the cathepsin B and L activity measured in this chapter, suggesting that the model can satisfactorily predict cathepsin B and L activity in beef brisket. The results also show that most of the released cathepsin B and L during *sous vide* cooking is retained within the meat fraction, as the amount in the cooking loss is only 4 % of that in the meat fraction. Interestingly, no tenderisation was observed in meat precooked at 46 °C, while significant decrease in shear force value was observed in meat precooked at 56 °C with increasing cooking time. Additionally, meat precooked at 46 °C and 56 °C has a similar cooking loss, while higher collagen solubilisation was observed in meat precooked at 56 °C compared to 46 °C. Furthermore, a marked decrease in final yield force was observed in meat precooked at 56 °C with increasing cooking time, suggesting that the weakening of collagen structure occurs under this precooking treatment. From this chapter (Chapter 4), it was concluded that higher cumulative cathepsin B and L activity did not result in tenderisation of meat indicating that cathepsin B and L do not contribute to the development of meat tenderness. This was supported by a significant correlation between cumulative cathepsin B and L activity and tougher meat ($r = 0.61$). On the other hand, meat tenderness appears to be correlated with collagen solubilisation ($r = -0.82$).

The change in meat tenderness during cooking is attributed to the thermal denaturation of meat proteins (Tornberg, 2005). Using differential scanning calorimetry (DSC), Chapter 5 shows that the thermal denaturation of meat protein consists of three peaks with T_{max} values of 56.0 ± 0.8, 66.8 ± 0.8, and 79.1 ± 0.8 °C for the first, second, and third peak, respectively, which is within the range reported in other studies (Chian et al., 2021; Vaskoska, Ha, Ong, Chen, et al., 2021). The first peak is attributed to light meromyosin (LMM) and heavy meromyosin subfragment-1 (HMM S-1), the second peak to heavy meromyosin subfragment-2 (HMM S-2), sarcoplasmic proteins, and collagen, and the third peak to actin. Several studies suggest that the toughening observed with increasing cooking temperature from 65 to 75 °C is attributed to collagen denaturation. In contrast, other studies suggest that collagen denaturation contributes to meat tenderisation (Bouton & Harris, 1972; Davey & Gilbert, 1974). Thus, to better understand the

effect of collagen denaturation on meat tenderness, particularly during prolonged cooking times, a model that can predict collagen denaturation in intact meat was developed in Chapter 5. The model was developed based on a three-protein model since the second peak also consists of two other proteins (HMM S-2 and sarcoplasmic protein) in addition to collagen. It was assumed that each protein (HMM S-2, sarcoplasmic protein, and collagen) has different thermal stability and denatures according to a first-order reaction. The ratio of each protein was calculated based on data reported by other studies. A satisfactory fit was obtained using the three-protein model (R^2 = 0.926), and the simulation using the kinetic parameters obtained showed that a marked increase in collagen denaturation was observed at 56 °C. This finding suggests that collagen denaturation may explain the meat tenderisation observed during prolonged cooking at 56 – 60 °C (Christensen et al., 2011; Fareh, 2018). Additionally, comparing collagen thermal denaturation with the activity of cathepsins B and L reveals that, at the temperatures where cathepsin B and L remain active, collagen is still in its native form. Since native collagen is less susceptible to proteolytic attack, this result indicates that cathepsins B and L may have a limited ability to degrade collagen during *sous vide* cooking.

Chapter 6 (Experiment 1) was designed to evaluate the effect of *sous vide* cooking at 46, 50, 53, and 56 °C for 0, 2, 6, and 12 h, representing different levels of cumulative enzymatic activity, before further cooking under two conditions favoring greater collagen solubilisation: 56°C for 48 h (commonly practiced in the kitchens) and 80 °C for 8 h (commonly practiced in the food industry). The results show no significant effect of precooking on meat tenderness compared to directly cooking at 56 °C for 48 h. This might be due to sufficient tenderisation already achieved by cooking at 56 °C for 48 h alone. Interestingly, the findings show that meat precooked at 46, 50, and 53 °C for 12 h and 56 °C for 6 and 12 h had a significantly lower shear force value compared to meat directly cooked at 80 °C for 8 h. However, faster tenderisation can be observed as early as 6 h with precooking at 56 °C. Although tenderisation with precooking at 46 °C, 50 °C, and 53 °C for 12 h (cumulative activity 2800 – 4630 nmoles AMC g^{-1}meat) may suggest the contribution of cathepsins B and L, faster tenderisation can be achieved by cooking at a low

cumulative activity (1270 nmoles AMC g^{-1}meat at 56 °C for 6 h), suggesting that cathepsins B and L may not be important in the development of meat tenderness during *sous vide* cooking, which is consistent with the findings in Chapter 4. Additionally, no improvement in cooking loss or collagen solubilisation was observed with the precooking treatments. The tenderisation observed with precooking at 56 °C is suggested to occur due to collagen denaturation and the weakening of the collagen structure during the precooking treatment. Additionally, the sequential protein denaturation and aggregation, achieved through sarcoplasmic denaturation during the precooking treatment followed by actin denaturation and myofibrillar aggregation during the second-stage cooking, might contribute to improved meat tenderness compared to the concurrent protein denaturation and aggregation in meat cooked directly at 80 °C for 8 h. This might also explain the tenderisation observed with precooking at 46, 50, and 53 °C for 12 h.

Based on this result, further studies were conducted to evaluate the effect of two-stage *sous vide* cooking with precooking at 56 °C for 6 h compared to conventional single-temperature *sous vide* cooking on meat tenderness, cooking loss, and its ability to reduce the total cooking time (Chapter 6, Experiment 2). The results show no significant difference in shear force value and cooking loss (particularly at temperature-time combinations that produce acceptable tenderness) between two-stage and conventional single-temperature *sous vide* cooking, especially when considering total cooking time. Several studies have reported improved tenderness in meat cooked using two-stage and multi-stage (three-stage) cooking compared to single-stage cooking. However, these studies did not account for total cooking time (Uttaro et al., 2019; Yao et al., 2023). On the other hand, Ismail et al. (2019) compared the effect of two-stage and single-stage *sous vide* cooking on the tenderness of beef *semitendinosus* under a similar total cooking time. However, they found conflicting results. For example, two-stage cooking at 49 °C for 6 h followed by 60 °C for 6 h and 49 °C for 6 h followed by 75 °C for 6 h, has a lower shear force value than their corresponding one-stage cooking at 60 °C for 12 h and 75 °C for 12 h. In contrast, two-stage cooking at 49 °C for 3 h followed by 60 °C for 3 h and 49 °C for 6 h followed by 70 °C for 6 h, has a higher shear force value than their corresponding one-stage sous vide cooking at 60 °C for 6 h and 70 °C for

12 h. This could be attributed to the high variability of the *semitendinosus* muscle. In this thesis, no significant difference in the shear force values of beef brisket was observed between two-stage and single-temperature *sous vide* cooking when considering the total cooking time. Previously, it was suggested that the greater tenderisation achieved with precooking at 56 °C for 6 h, compared to direct cooking at 80 °C for 8 h, is attributed to collagen and sarcoplasmic denaturation and the weakening of collagen structure during the precooking treatment. Thus, the lack of a significant difference in shear force values between two-stage *sous vide* cooking and single-temperature *sous vide* cooking when considering the total cooking time might be due to the fact that the tenderisation of beef brisket is mainly associated with the weakening of the collagen structure, such as through collagen solubilisation, which is also promoted at high cooking temperatures (second-stage cooking). This also suggests that the precooking treatment contributes to meat tenderisation during the two-stage cooking process mainly through an additive effect, rather than by causing a more significant weakening of the collagen structure or greater tenderisation during the second-stage cooking. Taken together, these results suggest that focusing only on the second-stage/ single-temperature *sous vide* cooking is sufficient to achieve tenderisation for tough meat cuts like beef brisket.

Consequently, the time required for beef brisket to reach consumer tenderness thresholds was determined based on conventional single-temperature *sous vide* cooking. The results show that at 60, 70, 80, and 90 °C, the times to reach consumer tenderness threshold are 20, 26, 12, and 8 h, respectively. The corresponding cooking losses at these temperature-time combinations are 33.8, 43.6, 44.0, and 45.6 % for 60, 70, 80, and 90 °C, respectively. This result demonstrated that *sous vide* cooking at higher temperatures, such as 80 and 90 °C, reduces the time required to achieve consumer tenderness threshold but also results in higher cooking loss. In contrast, *sous vide* cooking at 60 °C can produce lower cooking loss but requires a longer cooking time. Similarly, using process optimisation, Alahakoon, Oey, Bremer, and Silcock (2018) and Karki, Bremer, Silcock, and Oey (2022) reported that maximal tenderness with minimal changes in cooking loss and colour can be achieved with *sous vide* cooking at low temperatures, such as 60 °C, compared

to higher temperatures, such as 65 °C and 70 °C. They suggested that the optimal temperature-time combinations for *sous vide* cooking of beef brisket and beef short ribs are 60 °C for 24 h and 60 °C for 34 h, respectively.

Furthermore, it was found that *sous vide* cooking at 60 °C for 2 to 26 h resulted in cooking losses ranging from 29.2 – 34.0 %. Based on the kinetics of collagen thermal denaturation obtained from the three-protein model, *sous vide* cooking at 60 °C for 2 h already resulted in complete collagen denaturation. Additionally, Zielbauer et al. (2016) showed that *sous vide* cooking at 60 °C for 24 h produced complete meat protein denaturation, as evidenced by the disappearance of the meat's endothermic curve obtained through differential scanning calorimetry (DSC). Thus, it is suggested that the high cooking losses (42.8 – 45.7 %) observed with prolonged cooking at temperatures of 80 °C and 90 °C are due to factors other than protein denaturation, such as protein oxidation and aggregation. Consequently, to improve *sous vide* processing conditions, low-temperature *sous vide* cooking (60°C) could be combined with treatments that reduce cooking time (e.g., weakening the collagen structure), while high-temperature *sous vide* cooking (80°C and 90°C) could be used with treatments that reduce cooking loss (e.g., preventing myofibrillar oxidation and aggregation).

Apart from that, this chapter found that the development of meat tenderness during *sous vide* cooking is attributed to collagen solubilisation ($r = –0.54$), which supports the findings in Chapter 4. This aligns with results reported by other studies using beef *semitendinosus* and *biceps femoris* (Christensen et al., 2013; Naqvi et al., 2021).

7.2 Future work

Cathepsin B and L have long been implicated in the meat tenderisation that occurs during *sous vide* cooking (Laakkonen, Sherbon, & Wellington, 1970). This is mainly attributed to: (1) the increase in cathepsin B and L activity in the sarcoplasmic fraction of *sous vide*-cooked meat, which suggests the release of these enzymes from lysosomes, allowing them to come into contact with meat proteins, thereby has potential to contribute to their degradation; and (2) thermal stability. However, this thesis shows that cathepsin B and L may not be important in the development of meat tenderness during *sous vide* cooking. This conclusion is based on the fact that *sous vide* cooking at temperatures that favor high cumulative cathepsin B and L activity does not contribute to greater tenderisation compared to cooking at temperatures with low cumulative activity (Chapter 4 and Chapter 6, Experiment 1). The inability of cathepsin to contribute to tenderisation may be attributed to the following factors: (1) At temperatures where cathepsin B and L are still active, collagen remains in its native form. This may limit the ability of cathepsin B and L to degrade collagen (Chapter 3 and Chapter 5). (2) Heat treatment can cause the oxidation of myofibrillar proteins, which may reduce the ability of cathepsin to act upon them (Yu, Morton, Clerens, & Dyer, 2017). (3) Cathepsin might not be able to reach myofibrillar and connective tissue proteins, as low cathepsin B and L activity is observed in the cooking loss (Chapter 4), which suggests that cathepsin may remain inside lysosomes during *sous vide* cooking. The increase in their activity in the sarcoplasmic fraction observed after *sous vide* cooking might be due to the enhanced ease of extraction resulting from more efficient disintegration of muscle cells during homogenization caused by the heat treatment. Apart from that, an immunolabeling study by Berge et al. (2001) shows that although cathepsin is released due to lysosomal breakdown as a result of lactic acid injection, the released cathepsin is mainly located in the vicinity of the lysosome, which suggests their limited ability to interact with myofibrillar and connective tissue proteins. (4) The pH value of meat increases from pH 5.5 (raw) to pH 5.9 during *sous vide* cooking, which may reduce cathepsin B and L ability to degrade meat proteins (Chapter 6). Additionally, high pH and temperature may also favor the binding of small heat shock proteins

(sHSP) to the myofibrillar proteins. The binding of sHSP may offer protection to myofibrillar proteins from proteolysis by cathepsin B and L (Haslbeck, 2002; Lomiwes, Hurst, et al., 2014). Several studies have reported that no change in the 30 kDa and 28 kDa protein fragments was observed during *sous vide* cooking, which suggests limited myofibrillar proteolysis (Jeneske et al., 2024; Li et al., 2019).

Further studies should be conducted to support these hypotheses. First, histochemical studies should be performed to determine the localisation of cathepsins B and L in meat during *sous vide* cooking, which can confirm the release of these enzymes from lysosomes and their ability to come into contact with myofibrillar and connective tissue proteins. Additionally, a study to determine the association of sHSP with meat myofibrillar proteins during *sous vide* cooking is warranted to understand the role of sHSP in modulating meat tenderness during *sous vide* cooking. Furthermore, the application of antioxidants can be explored to understand their ability to reduce protein oxidation and promote cathepsins B and L activity during *sous vide* cooking. Moreover, studies should also be conducted using cathepsins B and L inhibitors to confirm the roles of cathepsins B and L in meat tenderisation during *sous vide* cooking (Hopkins & Thompson, 2001; Vaskoska, Ha, Ong, Kearney, et al., 2021).

The enhancement of meat tenderness with an increase in collagen solubilisation can be associated with the reduction in the residual collagen inside the meat after cooking. This is shown by a low significant correlation between the residual collagen inside the meat after cooking and the shear force value ($r = 0.31$) (Figure S2, Supplemental data). Apart from that, the increase in collagen solubilisation may indicate greater collagen degradation, which can result in the formation of a weaker gelatin structure inside the meat, thereby contributing to the improved tenderness. Several studies have demonstrated that increasing cooking temperature and time result in a decrease in the proportion of the γ-component (three cross-linked α-chains) of collagen, accompanied by an increase in the proportions of the β-component (two cross-linked α-chains), α-chains, and lower molecular weight collagen peptides. This change is linked to the degradation of both thermal-labile and thermal-stable collagen crosslinks, as well as collagen peptide bonds (Pan et al., 2018;

Wang et al., 2022). Therefore, further studies are needed to explore the changes in the proportions of the γ-component, β-component, α-chains, and lower molecular weight collagen peptides inside the meat during *sous vide* cooking to better understand their relationship with meat tenderness.

This study also shows that the kinetics of cathepsin B and L and collagen denaturation in beef brisket can be successfully modeled using consecutive reaction in series and a three-protein model, respectively. However, further studies should be conducted to understand the effects of different muscle types, animal species, pH, animal age, and post-mortem aging on the kinetic parameters. Additionally, the three-protein model developed in this study described collagen denaturation as a single first-order irreversible process. However, it is known that collagen denaturation can also be described using a two-step process: 1) reversible unfolding of the protein from its native form to an unfolded form, followed by 2) an irreversible transition of the unfolded form to the denatured form (Latorre et al., 2018a; Lumry & Eyring, 1954; Vyazovkin et al., 2007). Thus, further studies to model collagen denaturation in intact meat based on the two-step process are warranted. This could allow for a better understanding of the relationship between reversible collagen unfolding, enzyme activity, and meat tenderness.

This study also provides a guideline for temperature and time combinations that can be used by the meat industry to achieve consumer tenderness thresholds. However, the tenderness threshold in the guidelines is derived from a study using the beef *longissimus lumborum* muscle, which is a tender muscle with moderate collagen content (Liang et al., 2016; Torrescano et al., 2003). Additionally, in that study, tenderness was evaluated based on meat cooked at a single temperature (until the core temperature reached 70 °C). Thus, despite a large variation in shear force values, the variation in cooking loss may be smaller. However, for *sous vide* cooking, where meat is cooked at different temperatures for prolonged times, the development of tenderness is also accompanied by an increase in cooking loss. Several studies have shown that consumers will choose slightly tougher meat with high moisture content compared to tender but dry meat (Becker et al., 2016; Gámbaro et al., 2023). Thus, further studies should be conducted to develop a

tenderness threshold using tough meat cuts, which include consumer preferences for not only tender but also juicy meat. This can also be used to understand the consumer juiciness threshold.

7.3 Final conclusion

The kinetic models developed in this study have provided a better understanding of the relationships between cathepsin B and L, collagen denaturation, and meat tenderness during *sous vide* cooking. This thesis demonstrated that the development of meat tenderness during *sous vide* cooking was primarily attributed to collagen denaturation, collagen solubilisation, and the weakening of the collagen structure (as indicated by the reduction in final yield force). The enhancement of meat tenderness with an increase in collagen solubilisation was linked to the reduction in residual collagen inside the meat after cooking. Moreover, the increase in collagen solubilisation may indicate greater collagen degradation (a decrease in the proportion of the γ-component of collagen, accompanied by an increase in the proportions of the β-component, α-chains, and lower molecular weight collagen peptides), which can result in the formation of a weaker gelatin structure inside the meat, thereby contributing to improved tenderness. Further studies to determine the kinetics of collagen degradation during *sous vide* cooking could provide a better understanding of the mechanism of tenderisation due to collagen solubilisation.

This thesis also revealed that cathepsins B and L did not play a significant role in the development of meat tenderness during *sous vide* cooking. Although cathepsins B and L remained active during *sous vide* cooking, particularly at lower temperatures such as 46 °C, which allowed greater cumulative activity over time, precooking at this temperature did not promote greater tenderisation compared to precooking at temperatures with lower cumulative activity, such as 56 °C. Further studies are needed to investigate the underlying reasons for this, such as using immunolabelling to localise cathepsins B and L and examining the association of sHSP with myofibrillar proteins in meat during *sous vide* cooking.

This thesis also demonstrated that two-stage *sous vide* cooking did not offer benefits in terms of reducing total cooking time for tenderising tough meat cuts, such as beef brisket, compared to

conventional single-temperature *sous vide* cooking. When comparing the shear force values of meat cooked using single-temperature *sous vide* cooking with the tenderness threshold studied by Liang et al. (2016), it was found that *sous vide* cooking at higher temperatures, such as 80 and 90 °C, required less time to reach the consumer tenderness threshold but resulted in higher cooking loss. In contrast, *sous vide* cooking at lower temperatures, such as 60 °C, produced lower cooking loss but required a longer cooking time to achieve the consumer tenderness threshold. Thus, to improve *sous vide* processing conditions, low-temperature *sous vide* cooking could be combined with the treatments that reduce cooking time (e.g., weakening the collagen structure), while high-temperature *sous vide* cooking could be used with the treatments that reduce cooking loss by (e.g., preventing myofibrillar oxidation and aggregation).

Supplemental data

Table S 1 Predicted cumulative cathepsin B and L activity (obtained from Model 1, Chapter 3), fraction of denatured sarcoplasmic proteins, and fraction of denatured collagen (obtained from three-protein model, Chapter 5) in beef brisket *sous vide* cooked at 46, 50, 53, 56 °C for 2, 6, and 12 h.

Temperature (°C)	Time (h)	Cumulative cathepsin B and L activity (nmoles AMC g^{-1}meat)	Fraction of denatured sarcoplasmic proteins	Fraction of denatured collagen
46	2	804	0.0	0.0
	6	2418	0.1	0.0
	12	4636	0.2	0.0
50	2	937	0.2	0.0
	6	2487	0.6	0.0
	12	4139	0.8	0.0
53	2	949	0.7	0.0
	6	2042	1.0	0.1
	12	2802	1.0	0.1
56	2	808	1.0	0.2
	6	1213	1.0	0.6
	12	1271	1.0	0.8

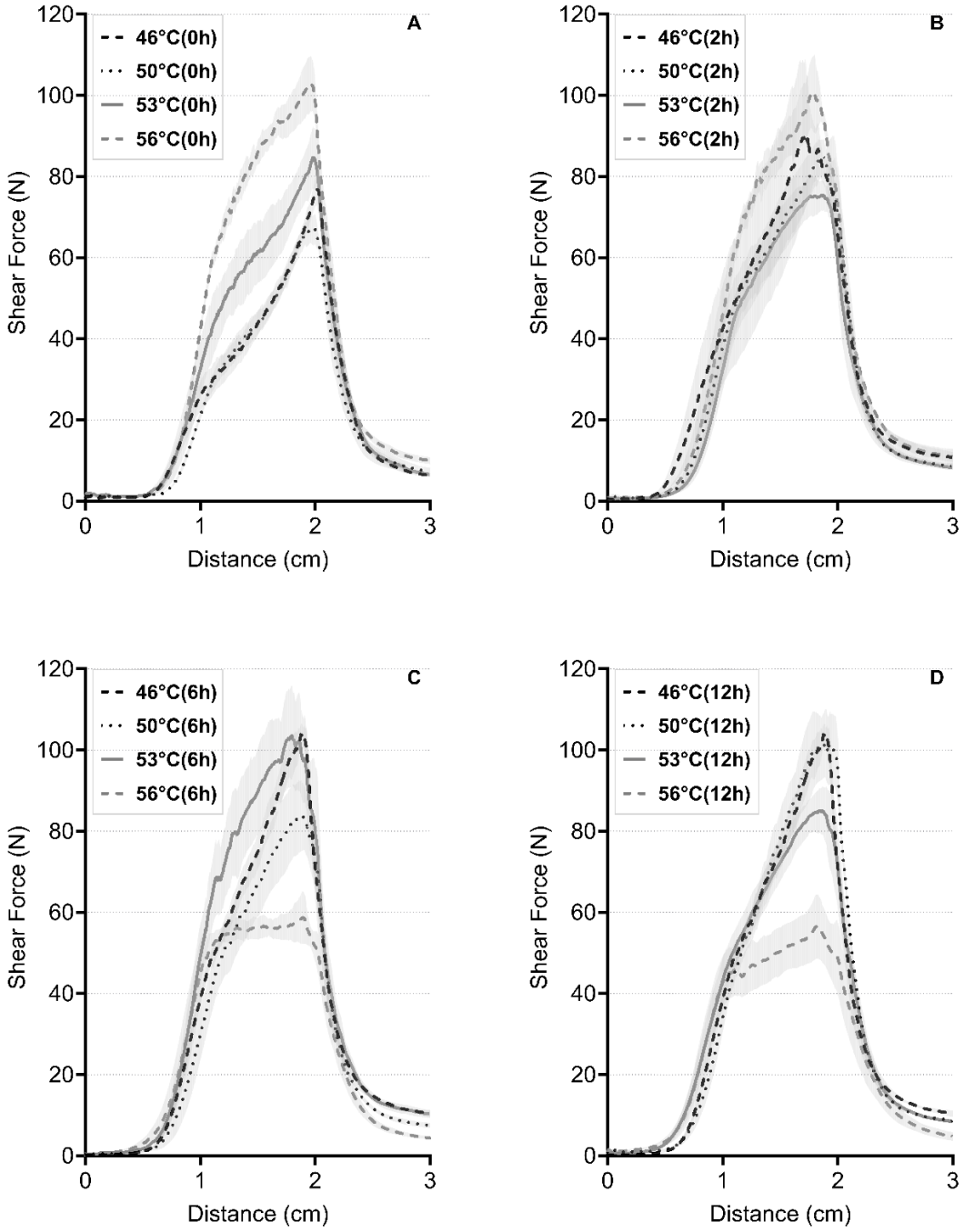

Figure S 1 Shear force deformation curve of beef brisket *sous vide* at 46, 50, 53, and 56 °C for A. 0 h, B. 2 h, C. 6 h, and D. Lines are means (n=3). Shaded area above and below a line represent SEM. The final yield force was defined as the force occurring at the position where the blade had completely cut through the meat sample (Møller, 1981). In this study, it occurred approximately at 1.866 cm of blade travel distance

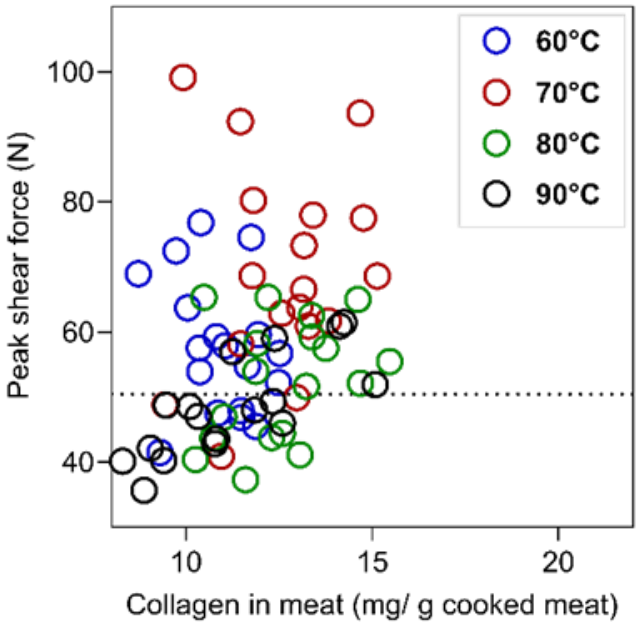

Figure S 2 Scatter plots of shear force value vs. collagen in meat residue (ColMF) of beef brisket *sous vide* cooked at different temperature and time combinations. N=72. Dashed line represents consumer threshold for tender meat (50.43 N). ($r = 0.31$, $p < 0.01$).

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