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The effect of proteolytic lysosomal enzymes and cathepsin B on collagen and connective tissue denaturation during long time low temperature cooking

Master Thesis

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Abstract

Low temperature long time (LTLT) cooking it has been seen to improve the palatability of meat by improving tenderness and juiciness as well as retaining a relatively pink colour in the meat. An important contributing factor in in tenderness is quality and amount of collagen rich connective tissue.

Collagen in connective tissue is thought to first denature and solubilized at temperatures above 70^{o}C. However earlier studies have found the improved tenderness between 50^{o}C and 65^{o}C to be a result of decreasing tensile strength in connective tissue. LTLT uses temperatures as low as 50^{o}C it is possible that proteolytic enzymes are active, which can potentially break down the insoluble part of collagen allowing for continued thermal denaturation of the rest of the protein.

The aim of this study was to investigate the effect LTLT cooking on proteolytic denaturation of connective tissue. To investigate the effect of LTLT on proteolytic activity, the study specifically considered cathepsin B activity of both the enzymatic mixture found in purified lysosomal extract and in pure cathepsin B from bovine spleen. The study used 55^{o}C, 60^{o}C and 65^{o}C and time intervals from 1 to 72 hours. To look at enzymatic effect as well as enzyme activity, the study observed the difference between thermal and enzymatic denaturation of isolated connective tissue and pure collagen with increasing time and temperature.

Cathepsin B activity reduced rapidly with increasing time and temperature for both purified lysosomal extract and the pure cathepsin B. Temperature was observed to have less influence on enzymatic activity than time.

Collagen and connective tissue denatured more when incubated together with enzyme compared to heat alone. Lysosomal extract resulted in the most distinguishable change in tissue texture for both substrates, though collagen was more denatured than connective tissue.

The study therefore concluded that though enzyme activity reduced quickly at temperatures between 50^{o}C and 65^{o}C, proteolytic activity still had some influence on thermal denaturation of collagen during LTLT cooking.

Contents

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1. Introduction

The busy lifestyle of modern western society has created a demand for quick, easy, nutritious food that tastes and looks great (Costa et al. 2007). This has created a niche market for convenience food which provide “ready to eat” and “heat and eat” food products.

The two main objectives when cooking meat are to obtain a palatable and safe product for consumption. Considering aspects of palatability; tenderness is highly sought after by consumers when buying meat (Aaslying et.al. 2007). In effect the American department for Agricultural Economics has found that customers are willing to pay a premium if they were assured a tender steak (Mintert et al., 2000).

However meat prices are on the rise, pushing food production companies to use more cost efficient meat cuts, such as chuck and foreshank, as their raw material. These cuts usually contain a higher amount of fat, cartilage and tendons compared to the high-end cuts such as filet steak, and require a completely different approach in meal preparation to produce the same level of high quality tender product (Schellekens, 1996; Resurreccio´n, 2003).

During cooking, heat works by changing intramuscular components such as sarcoplasmic proteins, muscle and connective tissue, altering their chemical and mechanical properties. The amount of change caused by denaturation of meat components can be controlled by varying time and temperature in the cooking process, hence by optimizing the applied cooking method a satisfactory tender product can be produced (Bramblett et al. 1959).

Sous vide is an up-and-coming cooking technique, and is the preferred technique in the cooking industry providing minimally processed “heat and eat” products. The technique works by vacuum packing the food in heat stable bags before cooking by steam in an autoclave or in a water-bath. It is a very heat stable technique compared to a conventional oven that heats by air, which has opened up a whole new direction for food preparation. This makes it perfect for cooking delicate foods such as custards.

In recent times however the most exciting exploration of the method has been within controlled low temperature long time (LTLT) cooking of proteins such as meat. The

combined LTLT *sous vide* technique has been found to retain flavour and juiciness as well as improving tenderness.

As cooking food at temperatures above 53^{o}C for 6 hours is considered microbiologically safe (Christensen, Tørngren and Gunvig, 2010), LTLT cooking provides the food production industry with a method to produce minimally processed heat and eat products on a large scale.

Research has found that LTLT cooking between 50˚C-65˚C retains a relatively uniform pink colour in the meat as well as a juicy and tender consistency (Beilken, Bouton, and Harris, 1986; Christensen et al., 2011). It has been suggested that this increase in tenderness is due to solubilisation of collagen from the connective tissue. However Davey and Niederer (1977) reported that collagen solubilizes at 70˚C, hence LTLT temperature should be too low to have any effect.

In order for the collagen to thermally denature and solubilize the heat labile part of the molecule needs to be made available. Ertbjerg, Larsen and Møller (1999) found that certain enzymes were able to denature the heat stable fractions of collagen, including proteolytic lysosomal enzymes such as cathepsins. Earlier research has found cathepsins to attack the stable cross-links in native collagen (Burleigh, Barrett, and Lazarus, 1974).

Christensen et al. (2011) showed that cathepsin B+L were active during LTLT cooking between 48˚C and 63˚C, it may therefore be possible for a combination of enzymatic and thermal denaturation during LTLT cooking.

Still the role played by proteolytic enzymes in denaturing collagen during LTLT cooking is unclear. The aim of this study was to further investigate proteolytic activity lysosomal enzyme under LTLT conditions and its possible effect on the heat-induced denaturation of collagen found in connective tissue.

The study will compare LTLT cooking of whole muscle against an *in vitro* study using specific enzymes and substrates. By using an *in vitro* approach the study will be able to look at the influence of the specific proteolytic enzymes cathepsin B, collagenase against the enzymatic mixture naturally found in purified lysosomal extract. Both isolated connective tissue and purified collagen from bovine meat will be used as substrate.

To get a better understanding of thermal versus enzymatic denaturation of connective tissue, the enzymes will be incubated with and without substrates.

The study will start by doing a literature search to get theoretical insight on current knowledge on the topic (chapter 2). It will continue by presenting the experimental methodology in three sections labelled raw materials and pre-experiments, experimental procedures and analysing procedures in chapter 3.

Analysis of results from the experimental procedures will be illustrated and commented upon separately (chapter 3) before being discussed against earlier literature (chapter 4).

2. Theory

2.1. Low temperature long time (LTLT) and *sous vide* cooking

The two main reasons for cooking are to improve palatability and to make the food safe for consumption.

The current definition of low temperature cooking is to use a lower than normal cooking temperature. Temperatures from 45-85°C can be used, however meat is most commonly cooked in a range between 50 – 60°C (Baldwin, 2012). In LTLT cooking the holding time can vary from some hours to several days, creating a spectre of manipulation possibilities of the food.

Sous-vide is French for "under vacuum"; *sous vide* cooking refers to cooking food in a vacuumized heat stable plastic bag or container (Figur 1) (Schellekens, 1996). By lowering the bag in to a tempered water-bath, the technique can use precise and controlled cooking temperatures. By using water as the heat transferring medium, the heat can efficiently and evenly transfer in to the food. After cooking the food is either served or rapidly chilled to temperatures below 4˚C to reduce chance of microbial development (Roca and Brugués, 2005).

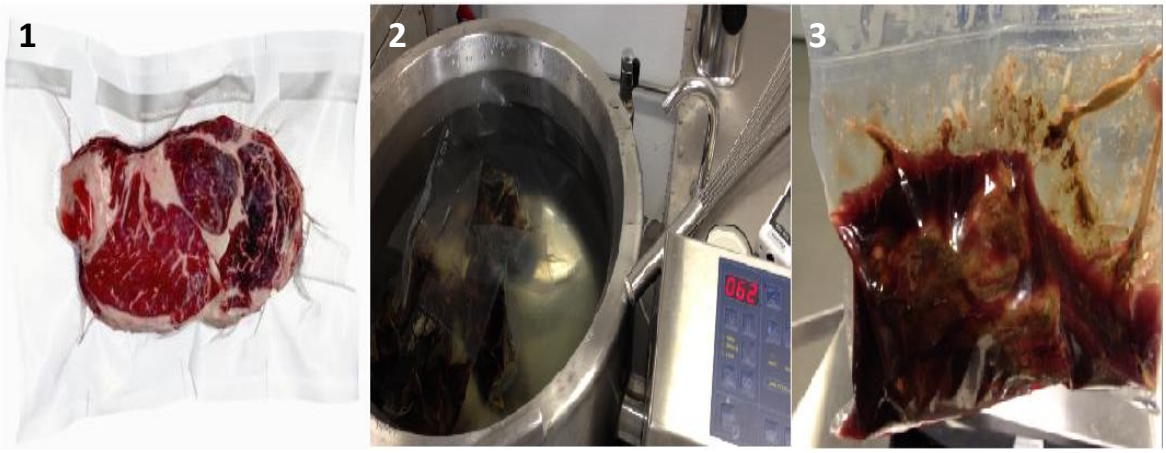


Figure 1. Sous vide cooking technique, 1. vacuum packed beef 2. Temperature controlled water-bath 3. *Sous vide* cooked beef.

The technique builds on George Pralus findings when experimenting with cooking *foie gras* (Keller et al., 2008). By using *sous vide* the liver retained more fat and kept its original appearance, which he found to improve the end products overall texture and taste experience. Since the 1990s the interest in *sous vide* has increased, resulting in extensive research in to the safety and shelf life of *sous vide* cooked food and in to time

and temperature combinations such as LTLT which is difficult to control with air based heating mediums (Mossel and Struijk, 1991; Ohlsson, 1994; and Schellekens, 1996; Christensen et al. 2011).

To date the technique has been deemed safe and provides the ready meal industry with a simple method for large scale meal preparation. The benefit of using a sealed vacuumized bag includes keeping in all juices exuded from the food, this may help keep flavour and improve texture and juiciness. The largest benefit of the technique is the temperature control as it creates a stable environment and allows for reproducibility (Keller et al, 2008), making it perfect to combine with LTLT cooking.

The LTLT *sous vide* influence on eating quality of meat has been greatly investigated (Laakonen, Sherbon, and Wellington, 1970; Bouton and Harris, 1981; Christensen, Purslow, and Larsen, 2000; Christensen et al., 2011 and 2012). The studies agree that LTLT *sous vide* cooking results in meat with an even pink colour as well as a juicy and tender texture.

The combined LTLT *sous vide* method uses temperatures above 53^{o}C making the food microbiologically safe (Christensen, Tørngren and Gunvig. 2010). The food may therefore be stored and distributed by the catering industry without concern for food safety. Church and Parson (1993) further press that the exclusion of oxygen can result in decreased chance of developing rancidity in oxygen sensitive food such as fats.

A disadvantage of LTLT sous vide is that the low temperatures do not result in browning or caramelisation of the meat surface as this requires temperatures above 120^{o}C (Whitfield and Mottram, 1992). However by browning the meat prior to or after LTLT cooking, the chef can overcome this obstacle.

2.2. Tenderness

One of the most important palatability qualities is tenderness, where consumers rate tenderness and beef quality above both nutritional value and cost (Smith at.al, 1987; Savell et al. 1989;Kukowski, et.al. 2005; Lusk and Fox, 2001). Hence obtaining tenderness is a high concern for ready meal producers, and producing consistently tender products is an important step in assuring customers continue to choose their product.

Tenderness can be defined as something easily cut or chewed. It is highly influenced by the condition of intramuscular factors such as muscle fibres and connective tissue. The connective tissue content play an important role in the varying degrees of tenderness between muscle cuts. Another important factor is the age of the animal upon slaughter, which determines the amount of heat stable cross links formed within the collagenous connective tissue (Lepetit, 2007).

Cooking changes physical properties of muscle tissue and can result in a more or less tender product. The physiological changes during cooking can be thermal or chemical/enzymatic denaturation of muscle proteins, which results in shrinking or denaturation and solubilisation of the muscle and connective tissue (Garcia-Segovia, Andres-Bello, Martinez-Monzo, 2007).

The relationship between cooking temperature and tenderness is not linear. As illustrated in Figure 2, Warner-Brazler (WB) shear force decreases when cooking at temperatures between 40˚C to 50˚C. After 50 ˚C the WB shear force drops, and an increase in tenderness continues towards 65˚C (Bouton & Harris, 1972; Davey and Gilbert, 1974; Christensen, Purslow, & Larsen, 2000). The change in shear force and sensation of tenderness in the LTLT region of 50˚C and 65˚C has been widely discussed in research and is attributed to physical and mechanical changes to muscle and connective tissue. This will be further discussed in the next sub-chapter.

The second rise in sheer force seen after 65˚C continues until temperature reaches 80˚C, where heating will result in reduced juiciness because of high cook loss as muscle fibres shrink, but also a decrease in WB shear force due to the solubilisation of collagen to gelatine (Light et.al. 1985).

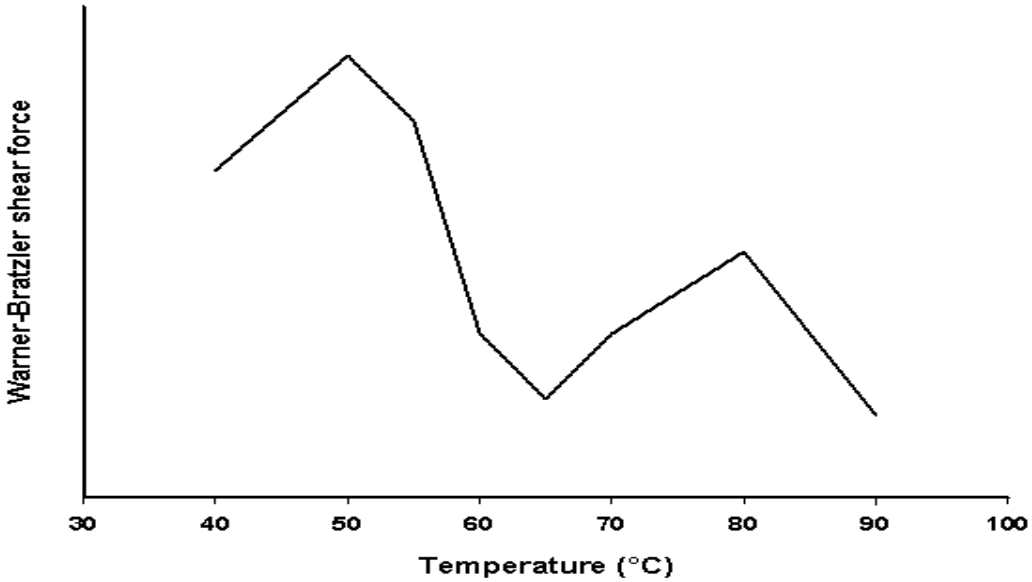


Figure 2. Illustration of a Warner-Brazler sheer force curve of meat heated to 40, 50, 55, 60, 65, 70, 80 and 90°C. (Christensen, 2012).

Research has found LTLT cooking to result in improved palatability factors of meat such as tenderness and juiciness (Vaudagna et al., 2002; Christensen et al, 2011 and 2012; Mortensen et al., 2012). However the theory behind the influence of LTLT cooking on changes in tenderness is widely debated, where there is some disagreement in the underlying physical and chemical reactions taking place.

As the technique uses low temperatures (<70°C) proteolytic enzymes are active, the research therefore discusses if the enzymes can play a part in protein denaturation during LTLT cooking (Christensen et al. 2011). As earlier studies have provided data mostly from empirical testing of whole meat, this study set out to perform an *in vitro* investigation of the tenderizing mechanisms that take place between LTLT temperatures, active enzymes and connective tissue.

2.3 Heat denaturation of proteins

Approximately 20% of meat is protein tissue (Aberle et al., 2001), which is mainly divided between the myofibrillar (50-55%), sarcoplasmic (30-34%), and connective tissue (10-15%) (Morrissey et al.,1987; Tornberg, 2005). Cooking works by denaturing these proteins with heat, and results in changing chemical and physical qualities.

Denaturation can be defined as an irreversible change in protein structure by heat and chemical agents (enzymes, acid, salts) resulting in a structural change altering physical or chemical qualities of the protein (Bender, 2006).

When heated, the sarcoplasmic proteins expand and form a gel (Davey and Gilbert, 1974) whilst myofibrillar and connective tissue contract and shrink (Tornberg, 2005).

The amount of denaturation mainly depends on temperature and time, the changes in denaturation can be followed by looking at physical factors such as tenderness.

The first increase in toughness between 40^{o}C and 50^{o}C (Figure 2) has been attributed to the denaturation and shrinkage of the myofibriller component myosin (Davey and Gilbert, 1974). The second rise in toughness between 65^{o}C and 80^{o}C however has been discussed conflictingly.

Martens, Stabursvik and Martens (1982) suggested it came to part as intramuscular connective tissue became denatured. Bouton and Harris (1972) and Christensen (2012) on the other hand contributed the decrease in toughness below 60^{o}C to reduction in connective tissue strength. Both studies found an age dependant difference in the relationship between decrease in toughness, maturity of the connective tissue, and cooking time required to reach desired tenderness. They therefore believed that the second ride in toughness was due to a change in the myofibriller component and not from the connective tissue.

The muscle fibres are thought to experience heat induced shrinkage of the transversely between 45-60^{o}C and longitudinally between 60-90^{o}C (Palka and Daun, 1999). An in vitro study performed by Christensen, Purslow and Larsen (2000) compared the tensile strength of low temperature treated perimysial connective tissue and single muscle fibres against the changes in toughness seen in low temperature treated whole meat. Through this experiment they confirmed that the decrease in toughness seen between 50^{o}C and 60^{o}C was due to mechanical changes to the perimysial connective tissue, whilst the increasing toughness above 60^{o}C was found to correspond with increasing breaking strength of the single muscle fibre that continues up to 90^{o}C.

Christensen et al. (2011) looked specifically at the effect LTLT cooking had on porcine meat; the study confirmed that cooking pork *latissimus dorsie (*LD) resulted in shrinkage of fibre diameters but no reduction of sarcomere length. The LTLT cooking

resulted in increased tenderness, which means that this shrinkage was too weak to result in a significant increase in toughness.

Collagen denaturation causes the connective tissue to first shrink and then solubilize in to gelatine. However in older animals and muscles used for heavy work heat-stable cross-links are formed, resulting in collagen that does not dissolve by thermal denaturation (Bailey, 1989). Previous studies have found a negative relationship between amount of solubilized collagen and shear values ($r= -0.704$, $p<0.01$) in beef *semitendinosus* cooked between 40-70^{o}C (Penfield and Mayer, 1975). They further found that solubilisation of collagen increased when meat was heated slowly to a low end temperature compared to a rapid heating rate to the same end temperature.

Other studies have found a positive relationship between increasing cooking time from 2 to 4 hours and amount of solubilized collagen (Dinardo et al., 1984). A quantities analysis was performed by Chrsitensen et al. (2011) where soluble collagen was measured in the LTLT treated meats cook loss. The study saw an increase in soluble collagen with increasing cooking time. However the amount of solubilized collagen was a low percent of the total collagen content of the meat. The authors hypothesises that the low amount seen in the cook loss may be because some of the solubilized collagen was held within the meat structure itself, hence the effect of solubilisation of collagen on LTLT meat tenderness could not be clarified.

Another study found that though increase in solubilized collagen had a positive relationship with tenderness for young bulls, the same relationship was not found with the increasing tenderness in LTLT cooked meat from older milking cows (Christensen et al. 2013). This suggests that the weakening of connective tissue may occur before or independently of heat solubilisation, this theory is strengthened by the observations made by Christensen, Purslow, and Larsen (2000) on reduction of tensile strength of perimysial connective tissue when cooked between 50^{o}C and 60^{o}C.

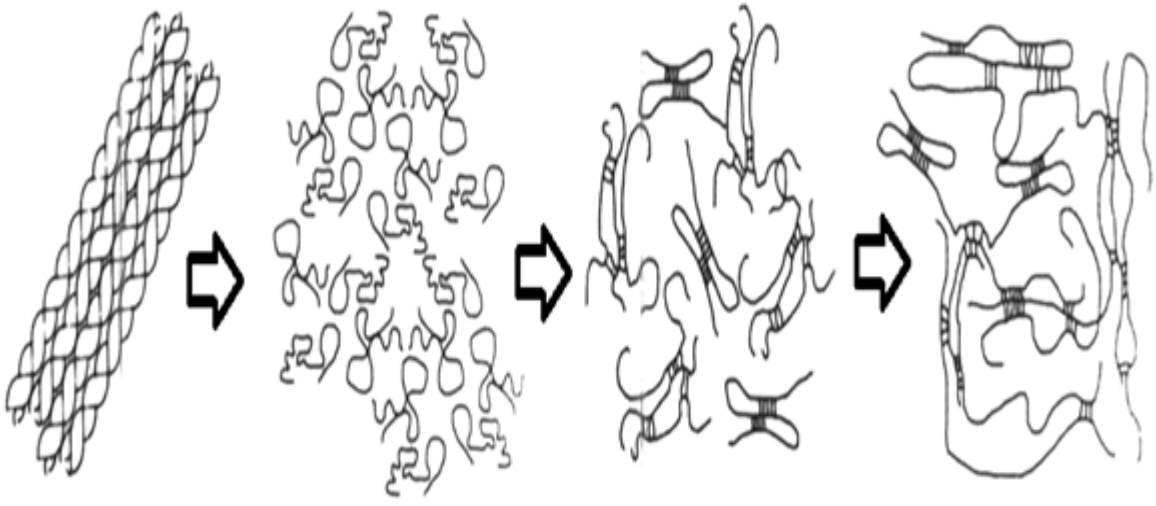
Heat induced aggregation of sarcoplasmic proteins occurs between 40^{o}C and 60^{o}C (Hamm, 1986), this results in a gel formation between the muscle tissue which glues structural components together (Davey and Gilbert, 1974). The sarcoplasmic gel is filled with proteolytic enzymes (Christensen, 2012); hence by working at low temperatures LTLT *sous-vide* may work not only by thermal denaturation of collagen, but also by other

factors such as enzymatic denaturation by proteolytic enzymes found in the sarcoplasmic proteins.

2.4. Collagen structure and denaturation

Collagen is an abundant structural protein found in animal tissue. The basic structure of collagen is a parallel left handed triple helix made up from three polyproline II-type helical peptide strands that coil in to each other (Shoulders and Raines, 2009). The polypeptide has a repeating XaaYaaGly sequence, where a glycine at every third position allows the helical molecule to pack tightly together. The Xaa and Yaa position can be a variation of amino acid (aa), however it is most frequently occupied by proline or hydroxylproline respectively, forming a repetitive ProHypGly sequence (Ramshaw, Shah, Brodsky, 1998). The hydroxyproline plays an important role in the stability of the quaternary structure by forming intramolecular hydrogen bonds (Jimenez, Harsch, and Rosebloom, 1973).

When cooking meat up to 64^{o}C the triple helical structure stays relatively stable, however when increasing the temperature further the helix starts to denature (Bailey, 1989). Initially the denaturation results in contraction and shrinking of the fibres, but with continued heating above 70^{o}C the collagen has been seen to solubilize resulting in a gelatine formation (Davey and Niederer, 1977)(Figure 3).



1. Fibrous collagen tissue
2. Heat denaturation and solubilization
3. Cooling and gel formation
4. Gelatin

Figure 3. Thermal denaturation of collagen in to gelatine (modified arrangements of images from Rose and von Endt, 1994)

Collagen denaturation can be divided in to three fractions depending on its ability to solubilize:

1. The heat soluble fraction also called Ringer's-soluble fraction. The heat labile part of the molecule can be quantified by the procedure described by Hill (1966)
2. The enzyme labile fraction, the fraction that is unprotected by the tightly coiled molecular structure has been found to be denatured by proteases (Snowden and Weidemann, 1978).
3. The unaltered fraction is the part of the collagen protected from denaturation by the tightly folded quaternary configuration. Without disrupting this configuration the enzymes are unable to attach and denature the triple helix (Snowden and Weidemann, 1978). The formation of intramolecular crosslinks is thought to strengthen this configuration and thereby increasing the total unaltered fraction.

Very little research has been done to investigate the effect of LTLT cooking on the denaturation of specific collagen fractions, meaning it is possible that by combining thermal and enzymatic denaturation over prolonged time more of the unaltered fraction will be denatured.

2.5. Proteolytic enzymes

Proteolytic enzymes are catabolic proteases. The four main proteolytic enzyme systems investigated involving the tenderization of meat include the proteasome (Coux, Tanaka, and Goldberg, 1996; Houbak, Ertbjerg and Therkildsen, 2008), the calpain system (Goll et.al., 1992; Huff-Lonergan, Zhang and Lonergan, 2010), the cathepsins (Agarwal, 1990; Sentandreu, Coulis and Ouali, 2002; Christensen et al., 2011) and the caspase system (Ouali et.al., 2006; Kemp and Parr, 2012). Other systems such as the collagenases have also been evaluated (Houbak et al., 2008), and it has been suggested that some of the enzyme systems work together (Baron, Jacobsen and Purslow, 2004). However the proteolytic activity of enzymes during LTLT cooking and its influence on connective tissue denaturation is not thoroughly studied.

Though some of the enzymes are dependent on release from cellular vesicles, such as cathepsins from the lysosomes, the presence of the enzymes in cook loss indicate the release of the enzymes in to the cytosol (Christensen et.al, 2011).

Theoretically LTLT uses temperatures so low that enzymes are still active, and could thereby result in the denaturation and weakening of muscle and connective tissue. Laakonen (1973) found that collagenases were active during prolonged heat treatment between 45^{o}C and 60^{o}C; however activity decreased with increasing heat. He further theorised that collagenase could work in symbiosis with other enzymes, where collagenase catalysed the unfolding of the collagen triple helix allowing other proteolytic enzymes to attach and weaken it.

Penfield and Mayer (1975) further demonstrated the connection between increased enzyme activity, by unspecified proteolytic enzymes, in cook loss from meat LTLT cooked between 40^{o}C and 70^{o}C. The reduction of toughness demonstrated by decreasing shear force was paralleled by increasing enzyme activity between 50^{o}C and 60^{o}C. The authors also commented that the activity was greater in cook loss from meat with a slow heating rate compared to that quickly.

Calpains are often excluded from LTLT tenderness investigations as it has been seen to completely inactivate after 55^{o}C, whilst active cathepsin B and L have been found even after 24 hours of heating at 55^{o}C (Christensen et al, 2011; Ertbjerg et.al. 2012). Upon further heating toward 70˚C the activity decreased after 1 ½hours. When investigating a purified form of cathepsin B and L from ostrich muscle, Van Jaarsveld, Naudé and Oelofsen (1998) found an optimal activity temperature of 50˚C with a steep decline in activity afterwards as temperature increased.

Cathepsin B and L work as endopeptidases, meaning they hydrolyse the peptide bonds in proteins (Agarwal, 1990), hence it may play a role in the weakening of collagen in connective tissue. Beltran, Bonnet and Ouali (1992) found that by incubating isolated connective tissue from calf and steers with cathepsin B, they could reduce the temperature needed for denaturation of connective tissue. The enzyme had a greater effect on the calf tissue than that of steer; they attributed this to the difference in amounts of heat stabile cross links between the two. It can therefore be hypothesised that cathepsin B plays a part in denaturing the thermally insoluble part of collagen, and helps to decrease denaturation temperature of connective tissue and decrease toughness.

To investigate the possibility of proteolytic influences on tenderness for cooking temperatures deemed safe for consumption, this study set out to specifically look at the enzyme activity across LTLT temperatures from 55˚C to 65˚C. In contrast to earlier studies which have looked at heating times from 1 to 32 hours, this study pushed past this and looked at enzyme activity from 1 to 72 hour of LTLT cooking.

2.6. Enzyme kinetics and enzyme assays

Enzyme kinetics is the study of the chemical reactions that are catalysed by enzymes. When using enzyme kinetics in research one often looks at the reaction rate and the influence of varying conditions. This allows the research to study the catabolic action of the enzyme, how it is controlled, and influence of outer parameters including drugs or changes to physical parameters such as temperature. The enzymes work by binding the substrate to an active site, the substrate is then transformed in to a product through a series of steps known as enzymatic mechanisms.

A method to investigate enzyme kinetics is through enzyme assays that measure reaction rate by studying reduction in substrate concentration or increase in product concentration over a set time. The changes in product concentration can be measured by absorbance, fluorescence or a colour change in the investigated medium.

For example the activity of cathepsin B and L can be investigated with the substrate Z-phe-arg-7-amino-methyl coumarin (Figure 4). After incubating the substrate with the enzyme for 10 minutes at 40^{o}C the end product, 7-amido-methyl coumarin can be measured fluorometrically at excitation and emission wavelengths 360nm and 540nm respectively (Christensen et al., 2011).

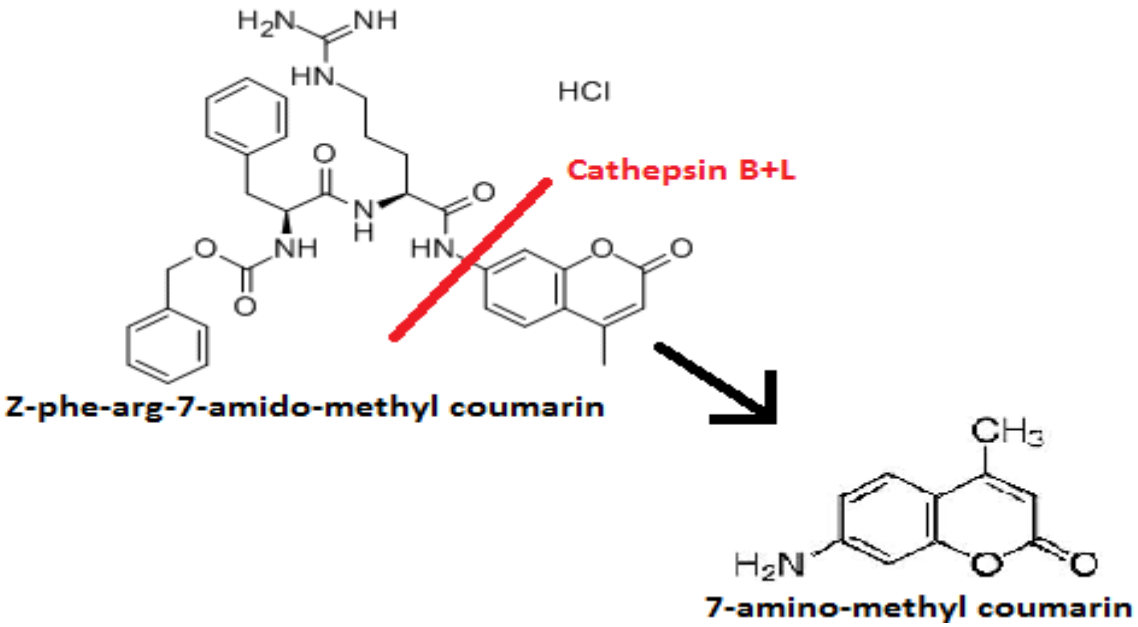


Figure 4. Enzymatic assay with cathepsin B with Z-phe-arg-7-amido-methylcomarine hydrochloride, 7-amino-methyl coumarin gives off fluorescence at emission and excitation wavelength 360nm and 540nm respectively.

The resulting absorbance can then be compared with a regression made from a dilution series of a standard that measures at the same excitation and emission, and enzymatic activity can be presented as amount of product produced per 10min per ml of investigated enzyme mixture (($^{nm}/_{10min}$)/ml). As the enzyme becomes less active or even inactivated the product accumulation will decrease, it can therefore be more interesting to measure enzyme activity than enzyme quantity.

3. Materials and experimental procedure

An overview of the experimental procedures performed can be seen in Figure 5. The raw bovine muscle was divided in to chunks before being LTLT cooked as either a whole muscle or as isolated connective tissue and purified lysosomal extract.

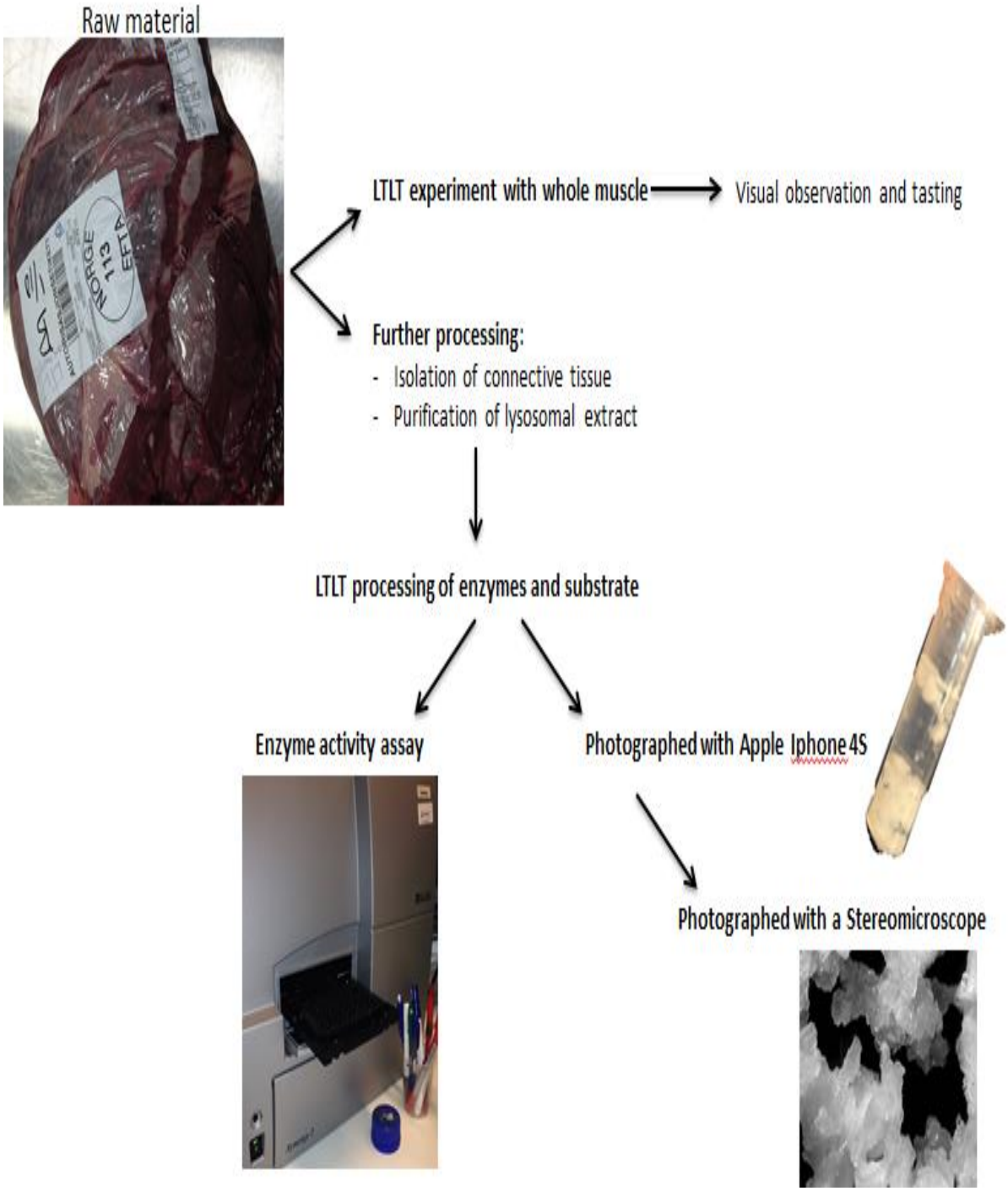


Figure 5. Flow diagram of investigative analysis

3.1. Raw material and pre- experiments

5kg *bovine trapezius* (BT) muscle was obtained from Nortura and kept at 4^{o}C until sampling. 7days post slaughter the meat was divided in to 50g portions, vacuum packed and then frozen at -80˚C for further use.

Cathepsin B from bovine spleen and collagenase from *clostridium histolyticum*, their specific substrates for enzyme assay investigation, Z-phe-Arg-AMC and Suc-Gly-Leu-Gly-Pro-AMC respectively, the standard solution 7-amino-4-methyl coumarin, and the experimental substrates insoluble Collagen from bovine achilles tendon were obtained from Sigma-Aldrich©.

The 0.5M MES buffer solution was made from MES anhydrous Biochemica from Aplichem GmbH.

3.1.1. Purification of lysosomal extract

Portions of 50g *bovine trapezius* muscle was thawed for 2 hours in a 4 ˚C fridge and then finely cut with a sharp knife before homogenizing with the Ulta Thurrax T18 at 6000rpm for 45sec in a homogenization buffer (100mM sucrose, 100mM KCl, 100mM Tris-HCl, 10mM sodium pyrophosphate, 1mM EDTA, pH 7.2). The meat was homogenized in a 1:2 meat to buffer ratio.

The homogenate was divided in to 8 tubes and centrifuged with a RC 5C Pluss centrifuge (Sorvall®) at 3000rpm for 10min. Discarding the pellet; the supernatant was transferred in to clean tubes and centrifuged at 5000rpm for 10min. Discarding the pellet, the supernatant was transferred in to clean tubes and centrifuged one last time at 12,900rpm for 27min.

The supernatant was then discarded and the remaining pellet was resuspended in 1ml solubilisation buffer (1mM EDTA, 0,2% Triton X-100, 50mM Sodium Acetate, pH 5.0) and kept overnight in a 4˚C fridge to extract enzymes.

The enzyme solution was transferred in to Eppendorf tubes and frozen at -70˚C awaiting further use.

Each round resulted in 8 Eppendorf tubes with approximately 1mlenzyme solution.

3.1.2. Isolation of connective tissue

Connective tissue was isolated from 50g portions of *bovine trapezius* muscle. The muscle was stored for 10days post slaughter and frozen at -80˚C, the meat was thawed in a 4˚C fridge before the isolating procedure.

Whilst the meat was moderately thawed it was cut in to small pieces and homogenized with 100ml cold 0.05M $CaCl_2$ by an Ultra Turrax T18 (IKA®) at 24000rpm for 45sec (Figure 6, picture 1). The homogenate was filtered through a sieve with holes of approximately 1mm (Figure 6, picture 2).

This procedure was repeated with a second 50g portion of meat.

The two portions were combined and cut in to small pieces, removing all obvious muscle fibre clumps, and rehomogenized together in 100ml cold 0.05M $CaCl_2$ and filtered through the sieve. This procedure was repeated 2 more times to make sure there were no obvious pink lumps of muscle tissue left. The remaining pellet was washed in 1%SDS for one hour to remove the residual muscle fibres (Figure 6, picture 3). After the hour it was centrifuged at 12.900rpm for 15min before being washed with 1%SDS and centrifuged a second time.

The last step was to rewash the residual pellet 3 times in cold 0.05M $CaCl_2$, sieve to remove moisture, and frozen at -20˚C.

The experimental procedures required approximately 27g connective tissue, the isolation procedure was therefore repeated 4 more times, using in total 500g meat resulting in approximately 30g isolated connective tissue.

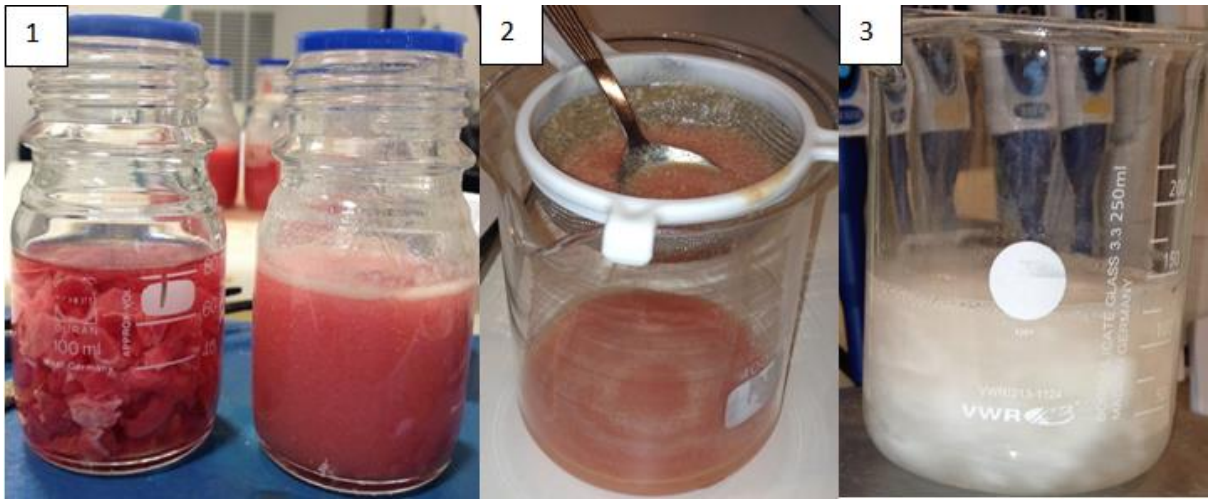


Figure 6. Steps of isolating connective tissue. 1. Homogenization, 2. Filtration with a sieve with holes approximately 1mm, 3. Washing with SDS.

3.1.3. Cathepsin B enzymatic assay for raw material

The enzymatic activity of the purchased cathepsin B and the purified lysosomal extract was quantified and compared before use in the main experiment to ensure that the test tubes would contain approximately the same initial amount of active enzyme.

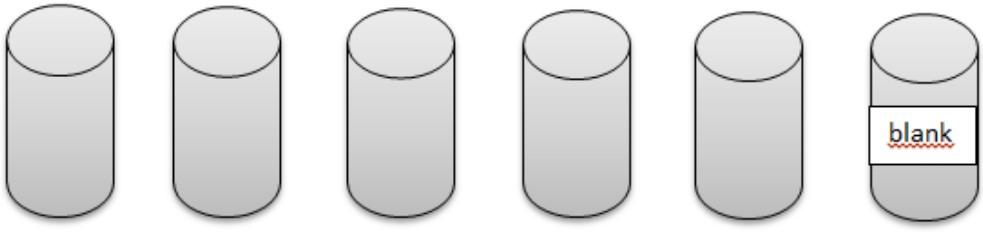
To make a cathepsin B stock dilution; 1.3mg of cathepsin B powder was mixed with 1.5ml MilliQ water, giving a concentration of 0.87mg/ml. This stock dilution was further diluted as 100µl enzyme stock in 10ml MilliQ, rendering a 0.087mg/ml cathepsin B dilution to be used in the experiment.

Cathepsin B enzymatic assay procedure was based on the technique described by Christensen et al. (2011). A small variation was made as the Cathepsin B stock was analysed in a dilution series (Figure 7) instead of with 15µl of investigative sample as was described in Christensen et al. (2011) technique.

The purified lysosomal extract on the other hand was examined in accordance with the technique using 15µl enzymatic sample.

Each sample in the dilution series was made in triplicate.

Enzyme and activation buffer (340mM sodium acetate, 60mM 100% acetic acid, 4mM EDTA, 0,1%Brij 35 (30%), pH 5.5+ 500µl 0.8mM DTT) was mixed and heated to 40˚C. 100µl substrate (Z-phe-arg-AMC) was added, mixed thoroughly and incubated for 10min at 40˚C. The potential reactions in the samples were halted by the addition of 1ml cold "stop" buffer (100mM NaOH, 30mM sodium acetate, 70mM 100% acetic vinegar, 100mM chloroacetic acid, pH 4.3).



Enzyme	15µl	12,5µl	10µl	7,5µl	5µl	0µl
Activation buffer	135µl	136,5µl	140µl	142,5µl	145µl	150µl
Substrate	100µl	100µl	100µl	100µl	100µl	100µl
Stop	1ml	1ml	1ml	1ml	1ml	1ml

Figure 7. Enzyme dilution series.

250µl sample was then added to a microtiterplate together with a standard dilution series and 3 blank samples, before analysis by Synergy 2 Fluroscan at excitation and emission wavelengths 355nm and 460nm respectively. The standard was made with 7-amino-4-methyl coumarin solubilized in DSMO and diluted with STOP-buffer.

Results were recorded by Gene5 software program and assessed in Microsoft excel.

The results showed a decreasing trend in absorbance (a.u.) from the most concentrated to the most diluted sample of Cathepsin B stock.

The activity of 15µL lysosome extract measured 117 a.u. which is lower but closest to the 5µl diluted Cathepsin B stock at 162 a.u. (Figure 8). In comparison to the bought purified enzymes it is approximately a third as active as the cathepsin mixture, and the mixture will therefore need to be further diluted before use in the experiment.

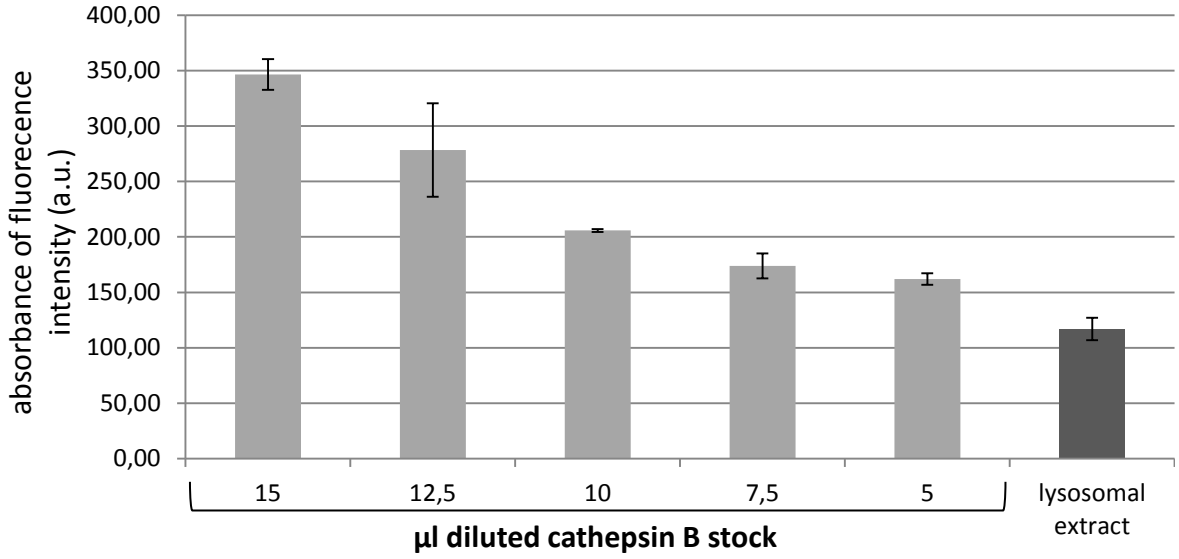


Figure 8. Absorbance (a.u.) recorded for enzymatic assay of Cathepsin B stock dilution series and purified lysosomal extract.

3.1.4. Collagenase enzymatic assay

An attempt to quantify the enzymatic activity of collagenase was conducted before using the pure form of the enzyme in the experimental procedure.

To investigate collagenase activity, the Cathepsin B activity procedure described by Christensen et al. (2011) was modified with a collagen specific substrate (Suc-Gly-Pro-Leu-Gly-Pro-AMC). The samples were analysed in a dilution series with triplicate samples for each dilution. The dilution series was produced as seen in Figure 7.

The first trial which used the same buffers as in the cathepsin B enzymatic assay was unsuccessful. Hence alterations to the activity buffer were made to suit collagenase enzyme sensitivities.

Firstly the EDTA was removed as this is a collagenase inhibitor (Hook et.al. 1971; Fullmer te al. 1972; Indra et al., 2005); secondly 0.5mM calcium was added to the buffer as the Sigma product description specified collagenase is active by four gram Calcium (Ca^{2+}) per mole enzyme. The third alteration was tried out by altering the pH from 5.5 to 7.3. As thiol functionalities is consequently reduced by sulphydryl groups causing irreversible inactivation of the collagenase (Hook et.al., 1971; Woessner, 1991), the last alteration tried out was to omit the addition of DTT.

No other steps in the Cathepsin B enzymatic assay procedure were altered.

Though no activity was registered for the purified collagenase, the purified lysosomal extract produced higher absorbance with the altered activation buffer 2 and 4 described in Table 1. To ensure that the increase in activity seen from lysosomal extract did not result from active cathepsin B breaking down the substrate, the collagen specific substrate was also tested against the cathepsin B stock solution for the various altered activation buffer. No increase in absorbance was recorded, suggesting that the lysosomal extract contains some other enzymatic activity that reacts with the substrate.

Table 1 Activation buffer composition according to alterations.

Buffer	pH	Buffer composition
Original	5.5	340mM sodium acetate, 60mM 100% acetic acid, 4mM EDTA, 0,1%Brij 35 (30%) + 500µl 0.8mM DTT
1	5.5	340mM sodium acetate, 60mM 100% acetic acid, 0,1%Brij 35 (30%), 0.01M CaCl, + 500µl 0.8mM DTT
2	7.3	340mM sodium acetate, 60mM 100% acetic acid, 0,1%Brij 35 (30%), 0.01M CaCl, + 500µl 0.8mM DTT
3	5.5	340mM sodium acetate, 60mM 100% acetic acid, 0,1%Brij 35 (30%), 100mM CaCl
4	7.3	340mM sodium acetate, 60mM 100% acetic acid, 0,1%Brij 35 (30%), 100mM CaCl

3.2 Experimental procedure

3.2.1. LTLT cooking of whole muscle

To get an initial idea of what happens in the meat during long time low temperature (LTLT) cooking, a pre experiment was conducted. 50g portions of bovine trapezius muscle were vacuum packed and placed in a water bath at heated at 60˚C or 65˚C. Samples were removed from the water bath at the time intervals 1, 6, 24, 30, 48 and 52 hours, where they were directly placed in ice water before being put in a 0˚C fridge.

After all samples had spent at least 12hours in the fridge the samples were opened and cut in half against the muscle fibres. The meat was tasted and visually assessed to consider changes in toughness, colour change, visually noticeable changes in the muscle fibres and cook loss.

3.2.2. In vitro LTLT experiment with enzyme and substrate

The study used four different conditions labelled A-D, though preformed at the same time each set of condition was labelled as a separate experiment. An overview of the cathepsin enzyme activity procedure can be seen in Table 2.

Table 2. Summarization of the 4 different experiments labelled by condition and defined by their contents.

Exp.	Cond.	Substrate	Active enzyme	Buffer
A	1	50mg purified connective tissue	-	1ml 0.5M MES
	2	50mg insoluble collagen	-	1ml 0.5M MES
B	1	-	Lysosomal extract	1ml 0.5M MES
	2	-	Cathepsin B	1ml 0.5M MES
C	1	50mg purified connective tissue	Lysosomal extract	1ml 0.5M MES
	2	50mg purified connective tissue	Cathepsin B	1ml 0.5M MES
D	1	50mg insoluble collagen	Lysosomal extract	1ml 0.5M MES
	2	50mg insoluble collagen	Cathepsin B	1ml 0.5M MES

Table 3. Experimental design (time/temperature) applied in the LTLT cooking of the four experiments. All conditions were made in triplicates (total 48 heat/temperature conditions *8 enzyme/substrate conditions (A-D) = 384 samples).

	Time intervals (hours)					
Temp.	0	1	12	24	48	72
55C˚	1	3	3	3	3	3
60C˚	1	3	3	3	3	3
65C˚	1	3	3	3	3	3

All the experiments described in Table 3 were conducted with the same procedure.

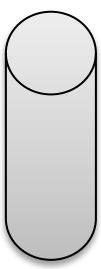
The procedure set out to use enzyme to give an initial activity of 6000 µU/ml. This was equivalent to 0.5ml lysosomal extract and 0.5ml of 0.01mg/ml cathepsin B. For substrate in experiment A, C and D (Table 2) either 50mg ($^{+}$/$_{-}$ 5mg) connective tissue or collagen were measured up a day in advance.

On the day of the experiment the buffer and active enzyme was added to the substrate and mixed with a VF2vortex mixer (Janke&Kunkel IKA®-Labratorytechnik) for 5sec before submerging the tubes in to the tempered water-baths. From this point the experiments were performed according to the design (time/temperature) seen in Table 3.

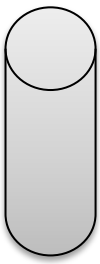
A 0hour sample representing each condition was given no heat treatment, but instead frozen at -70˚C directly after mixing the components.

The specific time intervals to take out samples were chosen on the basis of interest for use in ready meal products. The 12h interval was set as the second time interval on the basis of Laakonen, Sherbon, and Wellingtons (1970) findings that at least 6hours of heating was needed to result in a tenderizing effect.

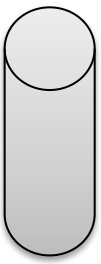
At each specific time point, samples were removed from the water-bath and split in to three new tubes. At 72 hours, the remaining connective tissue or collagen were collected and frozen at -70^{o}C for further investigation (Figure 9).



2* 200µL
Transferred to new 1,5ml tube to be used in cathepsin B enzyme assay



1* ca. 1ml
Transfer to a new 2ml tube to be used in soluble collagen investigation



Leftover solid
Orignial 2ml be used for visual analysis with Leica MZ8 stereomicroscope

Figure 9. Sample separation before freezing at -70˚C.

3.3 Analysing procedures

3.3.1 Visual observations

Changes that occurred during the experiments were noted down and photographed with an iphone 4S camera. For further investigation the leftover solid in the tubes were investigated with a Leica MZ8 stereomicroscope and photographed with a VisiCam 10.0 (VPR®).

The pictures were handled with Windows live photo gallery to adjust exposure and colour and optimize the clarity of the pictures. As the picture files were relatively large, they were readjusted by a factor of 0.0774.

3.3.2. Enzyme activity assays

The cathepsin B enzymatic assay was measured fluorometrically in triplicate for each sample; the procedure was performed as descried by Christensen et al. (2011). However instead of using 15µl, 30µl was mixed with 120µl of activation buffer (340mM sodium acetate, 60mM 100% acetic acid, 4mM EDTA, 0,1%Brij 35 (30%), pH 5.5, + 500µl 0.8mM DTT) and heated at 40˚C to equalize the temperature of the sample.

100µl substrate (12,5µM Z-Phe-Arg-Nmec) was added, the samples was mixed before being incubated at 40˚C for 10min. The enzymatic action was stopped by adding 1ml cold STOP-buffer (100mM NaOH, 30mM sodium acetate, 70mM 100% acetic vinegar, 100mM chloroacetic acid, pH 4.3).

250µl sample was added to the wells in a microtiter platter in triplicate before measuring absorbance at emission wavelengths 355nm and 460nm respectively.

The standard was made with 7-amino-4-methyl coumarin solubilized in DSMO and diluted with STOP-buffer, the standard dilution series was used to produce a regression. A blank sample was produced by adding 150µl of activation buffer instead of 30µl sample.

A collagenase assay will be attempted on samples containing purified lysosomal extract following the enzymatic assays producing results in the pre-experiment (3.1.4).

The samples will be compared against the same regression as lysosomal extract as it should measure absorbance at the same excitation and emission.

An after experiment was performed investigating interference of solubilized collagen on the cathepsin enzymatic assay. The same procedure as described for cathepsin enzymatic assay was followed in a double triplicate for the samples incubating 50mg collagen with either lysosomal extract or pure cathepsin B and a random selection of other samples from the main experiment.

After heating to 40˚C half of the samples were incubated with and half without 100µl substrate (12,5µM Z-Phe-Arg-Nmec).

250µl sample was added to the wells in a microtiter platter in triplicate before measuring absorbance at emission wavelengths 355nm and 460nm respectively.

3.4. Enzyme activity calculations

The different conditions were investigated in triplicate during the experiment and further in triplicate during the investigation and lastly measured in triplicate in the microtiter plate. This means that each condition produced 27 measurements for the enzyme activity investigation.

Enzyme activity was measured fluorometrically at excitation and emission wavelengths of 355nm and 460nm respectively. An average and standard deviation for all the absorbance for each condition was produced.

An average absorbance for the blank sample on the corresponding microtiter plate was deducted from the mean condition absorbance.

The resulting absorbance measurements were further put in a regression equation produced by the 7-amino-4-methyl coumarin (Sigma) dilution series with known concentrations.

Data was in Gene5 software and processed in Microsoft Excel 2010© and Minitab15 statistical software (Minitab Inc. ©).

3.4.1 Regression equation

A standard regression equation is seen as

$$\mathbf{y = ax - b} \qquad \textbf{Equation 1}$$

Where the dependable variable *y* is a linear combination of the independent variable ax which constitutes the slope of the regression and unknown parameters *b* which is the point where the line crosses 0.

In total 18 *7-amino-4-methyl coumarin* dilution series were produced. By combining the data the following regression equation was made:

$$\mathbf{y = 1.1327x - 7.6803} \qquad \textbf{Equation 2}$$

R^2=0.9991

However as we already have removed the disturbance of unknown parameters by deducting the blank sample absorbance, and by further assuming that negative absorbance is physically impossible the equation was rewritten to intercept at 0, giving the equation:

$$\mathbf{y = 1.0794x} \qquad \textbf{Equation 3}$$

R^2= 0.9954

As absorbance (a.u) represents the dependant variable *y,* the equation can be rewritten to find the relative enzyme activity (nmol/10min) by expressing the equation for x.

$$\boldsymbol{x = \left(\frac{nmol}{10min}\right) = \frac{a.u.}{1.0794}} \qquad \textbf{Equation 4}$$

3.4.2 Concentration equation

The relative enzyme activity over time (nmol/10min) can now be used to find the relative concentration of active enzyme in the samples from each condition. To present the relative enzyme activity, a unit (*U*) was defined (Equation 5).

$$\boldsymbol{U = nmol/10min} \qquad \textbf{Equation 5}$$

For practical application, the *U* was redefined as µmol/min. The equation was therefore modified by a factor of 1000 to make nmol in to µmol. Secondly the equation was modified by a factor of 10 which made 10min in to just min.

$$\boldsymbol{U = \frac{\left(\frac{\text{nmol}}{\text{10min}}\right)}{1000*10} = \frac{\mu mol}{\text{min}}} \qquad \textbf{Equation 6}$$

To further be able to compare the samples, the equation was made to find U per ml of sample. The enzyme activity was therefore divided by the amount of experimental sample (30µ) investigated during the enzymatic assay procedure in each microtiter plate well (250µl), hence:

$$\boldsymbol{\frac{U}{ml} = \frac{\mu mol}{\text{min}} / \frac{30}{250}\mu l} \qquad \textbf{Equation 7}$$

As it is more useful to compare activity per ml of sample compared to per µl sample the equation was modified by a factor of 10^-3. However as this is done on both side of the fraction bar it eliminates itself and the equation stands as it is but now in ml instead of µl.

To make the equation more practical fore use, the 30/250ml was flipped and the equation rearranged to have only one fraction bare:

$$\boldsymbol{\frac{U}{ml} = \frac{250}{30} ml * \frac{\mu mol}{\text{min}}} \qquad \textbf{Equation 8}$$

As the measurements are very small, the unit was converted from *U* to *µU* by multiplying by a factor of $10^{\wedge 6}$.

$$\boldsymbol{\frac{\mu U}{ml} = \left(\frac{250}{30} ml * \frac{\mu mol}{\text{min}}\right) * 10^6} \qquad \textbf{Equation 9}$$

4. Results

4.1. Whole muscle LTLT investigation

Some of the palatability factors assessed for LTLT treated bovine trapezius muscle did not change across the LTLT cooking; including a relative pink colour and sensation of juiciness. Whilst others factors such as sensation of tenderness and the physical structure of cook loss, changed gradually with time and temperature.

Sensory evaluation found a decrease in toughness with increasing time for meat cooked at 60˚C and 65˚C. After 6 hours the meat became easier to chew, and meat heated for 24-52hours was considerably more tender compared with the 1 hour sample. The most profound change in tenderness was experienced after 52 hours.

Though similar changes were seen at both temperatures, the meat heated at 65˚C for more than 36 hours presented the most noticeable physical alterations (Figure 10).

As seen in the figure the structure of perimysial connective tissue was still visible between fascicles at 36 hours. From 36 to 48 hours the shrinkage in myofibrillar tissue created a tighter cluster like structure. On a closer look one can also observe a reduction invisible perimysial tissue around the bundles suggesting denaturation of connective tissue.

The greatest textural change came between 48 and 52 hours. Cooking the meat for 52 hours resulted in markedly less resistance when chewing and muscle fascicles were easier to tear apart. At this point the connective tissue was barely visible between and colour had become duller.

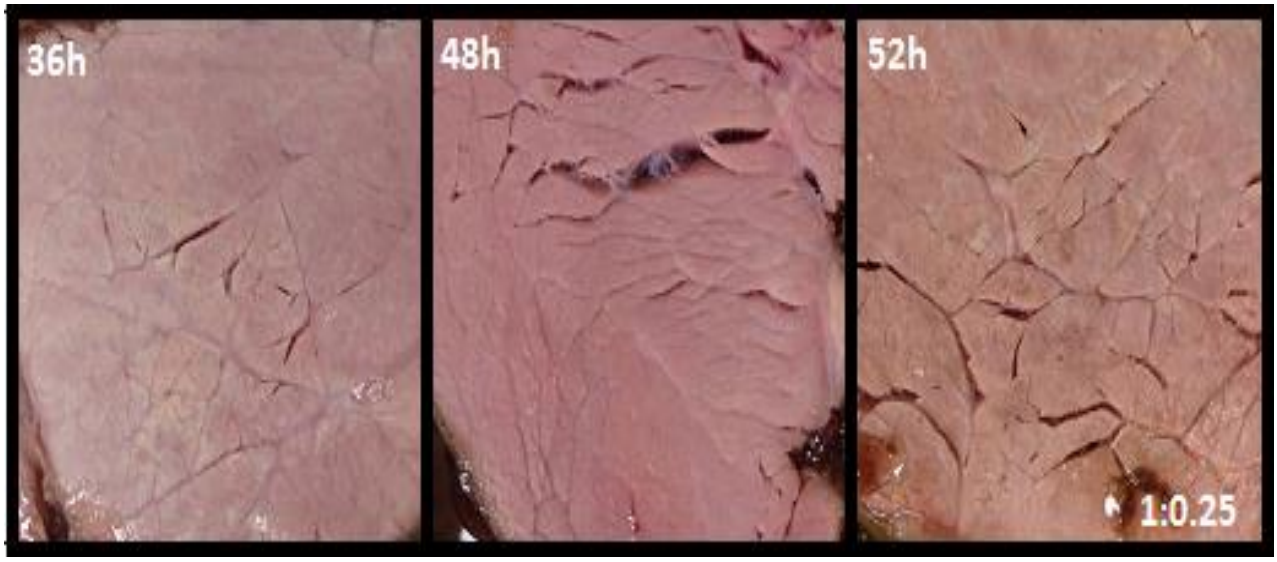


Figure 10. Visual changes observed in LTLT treated bovine trapezius muscle heated to 65˚C for 26, 48 and 52 hours.

Both the meat heated at 60 and 65˚C resulted in cook loss in the sous vide bag. When withdrawing the meat from heat after 48 hours and leaving it in a 4C˚ refrigerator for 24hours, a consistency change occurred in the cook loss(Figure 11).The 65˚C cook loss had become completely solidified in a gel matrix whilst the 60˚C cook just had some formed within the liquid.

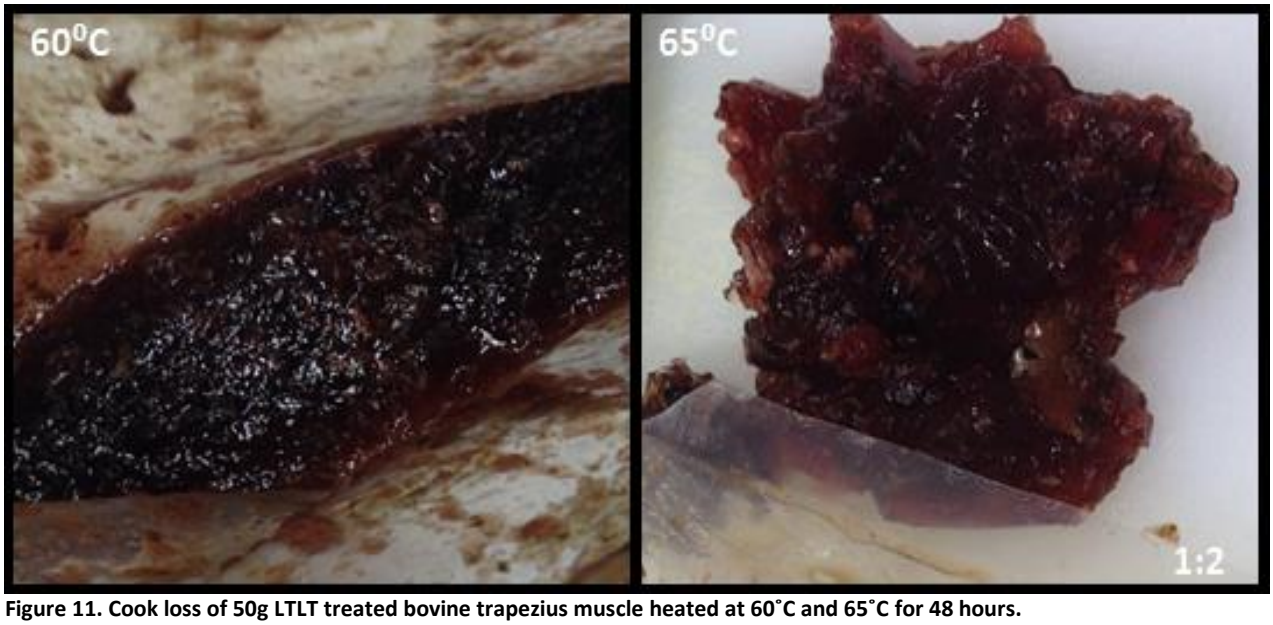


Figure 11. Cook loss of 50g LTLT treated bovine trapezius muscle heated at 60˚C and 65˚C for 48 hours.

4.2. Enzymatic assays

4.2.1. Cathepsin B activity

Experiment A

In the two conditions incubating substrates without enzyme, there was great differences in measured cathepsin B activity from LTLT treated isolated connective tissue (A1) or collagen (A2).

After subtracting the blank from the 0 hour samples, both A1 and A2 measured negative cathepsin B activity (Figure 12). A1 samples continued to measure negative activity across time and temperature, the only exception was seen for 48 hours samples cooked at 65˚C. There were no significant changes were seen between A1 measurements (Appendix 1, p. 1).

A2 showed a steady increase in measured absorbance with time and temperature. The increase between 0 to 12 hour at 60˚C and 65˚C, and from 0 to 48hours at 55˚C proved significant ($p<0.05$).

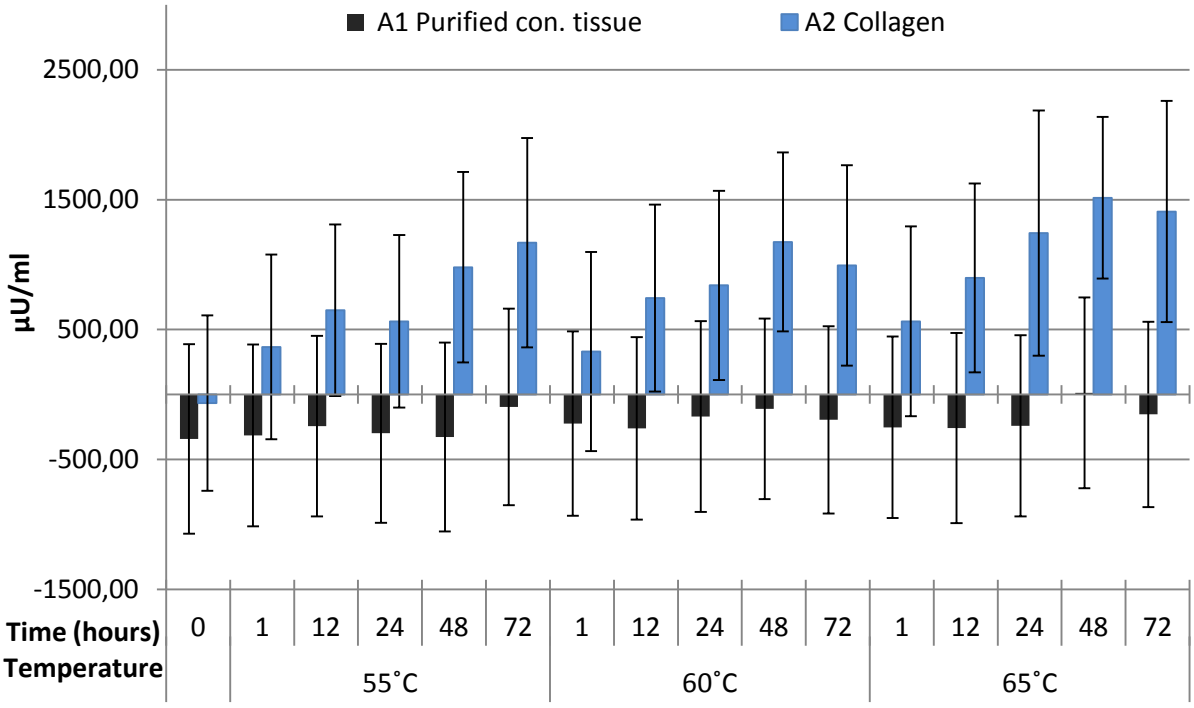


Figure 12 .A graphical illustrations of the mean cathepsin B activity measurements based on recorded absorbance in samples incubated with substrate, purified connective tissue (A1) or collagen (A2), without any enzyme added.

Experiment B

In the samples where purified lysosomal extract (B1) and cathepsin B (B2) were LTLT treated without substrate, cathepsin B activity decreased with time and temperature (Figure 13). The reduction between 0 and 1 hours of heating, was significant for both enzymes at all temperatures ($p<0.05$) (Appendix 1, p. 2).

As illustrated in Figure 13, the reduction in cathepsin B activity was gradual for B1. There was a significant reduction between 1 to 12 hours for all temperatures, and 12 to 24 hours for samples heated at 55˚C and 60˚C also proved significant. Though the activity continued to decrease, there was no significant change after 24 hours.

For B2 samples there was a large reduction between 0 and 1 hour and a stable low activity with no significant changes between 1 and 72 hours.

In comparison, B1 samples started off with a significantly higher activity than the B2 samples (Table 4). Due to the more gradual reduction of cathepsin activity, B1 samples continued to have a significantly higher activity after 12 hours for the samples heated at 55 and 60˚C. After this time point there was no more significant difference between B1 and B2 with increasing time or temperature.

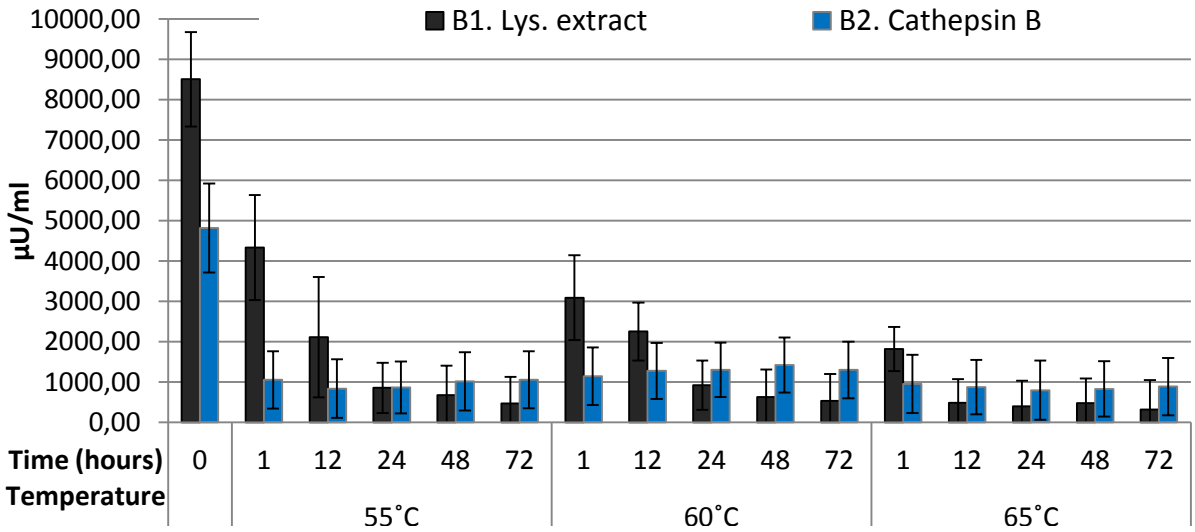


Figure 13. Mean values cathepsin B activity of LTLT incubated samples containing enzyme, lysosomal extract (B1) or Cathepsin B, and 0.5 MES buffer.

Table 4. Mean and significant difference (*p*) C1-C2 LTLT treated at 55°C, 60°C and 65°C for 1 to 72 hours.

Time (hours)	1		12		24		48		72	
µU/ml	mean	*p*	mean	*p*	mean	*p*	mean	*p*	mean	*p*
55˚C	3282.17	0.0001	1275.69	0.0001	180.14	1	-342.42	1	-586.44	1
60˚C	1951.15	0.0001	977.10	0.0001	-382.62	1	-799.89	1	-763.18	1
65˚C	863.87	0.0001	-386.72	1	-396.21	1	-354.56	1	-569.05	1

Experiment C

In samples incubated with isolated connective tissue, cathepsin B activity was significantly lower in samples incubated with lysosomal extract (C1) compared to those incubated with Cathepsin B (C2) at 0 hours (Appendix 1, p.3). Figure 14 shows progression of mean cathepsin B activity from 0 to 72 hours for all temperatures.

The reduction rate in cathepsin B activity was temperature dependent for C1 samples. Both samples heated at 55°C and 60°C had a significant reduction between 0 and 1hour as well as 1 and 12 hours, whilst 65°C had a larger reduction between 0 and 1 hour and no significant reduction afterwards.

Between 0 and 1 hour the reduction in enzyme activity reduced significantly in C2, after this point activity stayed relatively steady between 1 and72 hours with no significant changes.

C2 had significantly higher activity than C1 at 0 hours. The reduction in enzyme activity was more rapid for C2, resulting in a significantly lower activity in C2 compared with C1 after 1 hour at 55 and 60˚C (Table 5). After 1 hour there was no significant difference in enzyme activity with increasing temperature or time.

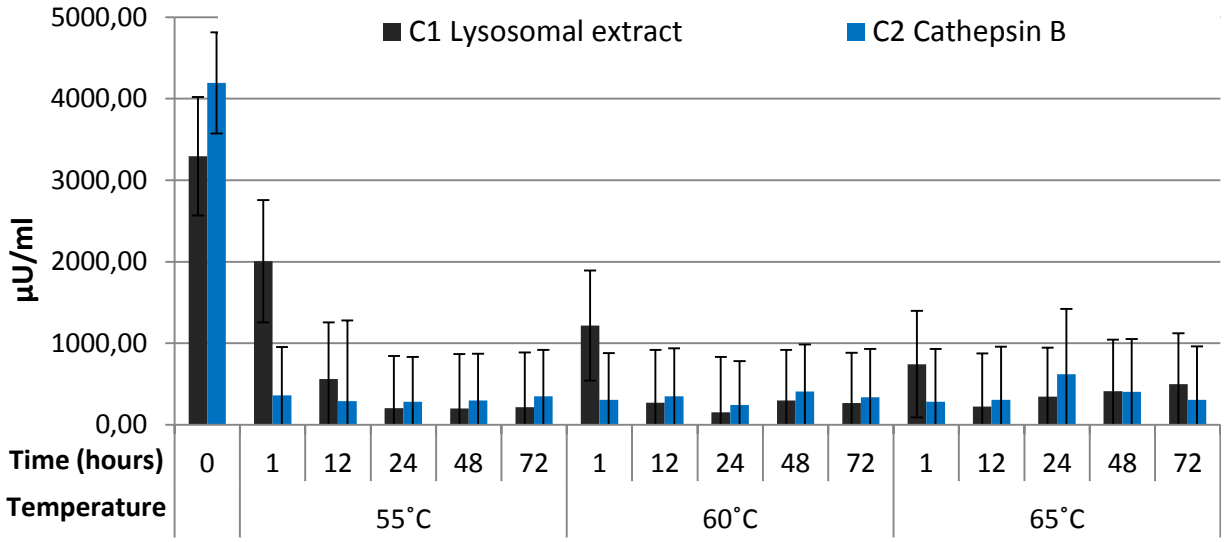


Figure 14.. Mean values of cathepsin B activity in samples containing enzyme, isolated connective tissue and MES buffer.

Table 5. Mean and significant difference (*p*) between C1-C2 LTLT treated at 55°C, 60°C and 65°C for 1 to 72 hours.

Time (hours)	1		12		24		48		72	
µU/ml	mean	*p*	mean	*p*	mean	*p*	mean	*p*	mean	*p*
55˚C	1644.2	0.0005	271.6	0.0005	-77.2	1	-97.22	1	-134.4	1
60˚C	910.7	0.0002	-75.8	1	-90.1	1	-110.09	1	-70.1	1
65˚C	461.8	0.4	-84.4	1	-273.1	1	7.15	1	193.0	1

Experiment D

Cathepsin B activity in samples where lysosomal extract (D1) and Cathepsin B (D2) was incubated with collagen had a significant ($p<0.05$) reduction in cathepsin B activity between 0 and 1 hour at all temperatures (Appendix 1, p. 4).

D1 has a second significant reduction between 1 and 12 hours at 60˚C.

D2 on the other hand had a significant increase in activity between 1 and 72 hours at 65˚C. No other differences in measured activity proved significant for D1 or D2.

At 0 hours D2 had significantly higher activity than D1, where both conditions had a significant reduction between 0 and 1 hour at all temperatures (Figure 15).

The decrease in activity was more rapid for D2 compared with D1, which results in D1 having significantly higher activity at all temperatures at 1 hour (Table 6). There was also a significant difference at 12 hours for samples heated at 55°C; no other temperature or time resulted in significant difference.

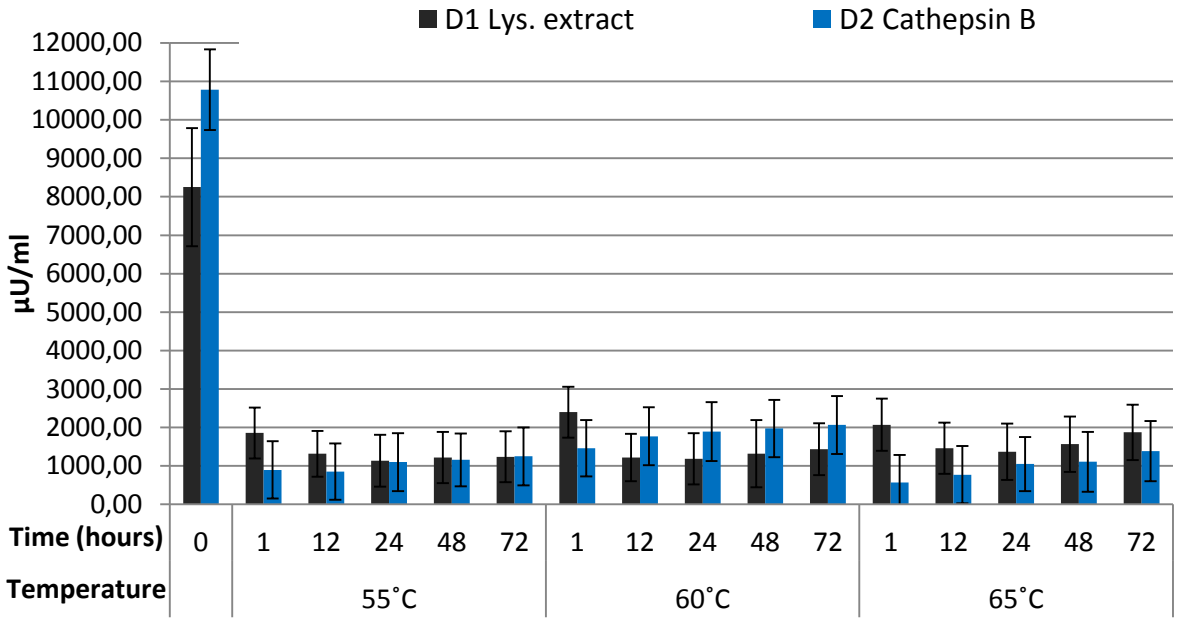


Figure 15. Mean enzyme activity in samples containing insoluble collagen in solution with enzyme and MES buffer.

Table 6. Mean and significant difference (*p*) D1-D2 LTLT treated at 55°C, 60°C and 65°C for 1 to 72 hours.

Time (hours)	1		12		24		48		72	
µU/ml	mean	*p*	mean	*p*	mean	*p*	mean	*p*	mean	*p*
55˚C	961.1	0.0001	458.7	1	36.1	0.5	61.9	0.4	-10.9	0.7
60˚C	938.9	0.0001	-549.8	1	-704.0	1	-651.3	1	-629.6	1
65˚C	1498.3	0.0001	687.7	0.07	315.4	1	458.3	0.9	486.3	0.9

Interference of heat denatured substrate

Figure 16 and 17 was produced to compare enzyme activity in samples containing the same substrate, and therefore address the increase in activity seen in experiment A and if heat denatured substrate could affect recorded enzyme activity.

At 0 hours the enzyme activity in both conditions containing enzyme, either lysosomal extract or Cathepsin B, was significantly higher than the condition without enzyme for both the experiment using isolated connective tissue and insoluble collagen.

As seen in experiment A (Figure 12), the condition with isolated connective tissue and no enzyme (A1), had negative absorbance and could be seen as not having any activity present.

As A1 never measured any cathepsin B activity, the conditions with isolate connective tissue and lysosomal extract (C1) and pure Cathepsin B (C2) measured overall higher activity (Figure 16). After 1 hour the difference was insignificant between A1 and C1, but was significant between A1 and C2 at all temperatures. The difference between A1 and C1 at 55˚C up to 24 hours, and at 60 and 65˚C to 12 hours also proved significant.

No other differences between A1 and C1 or A1 and C2 were significant.

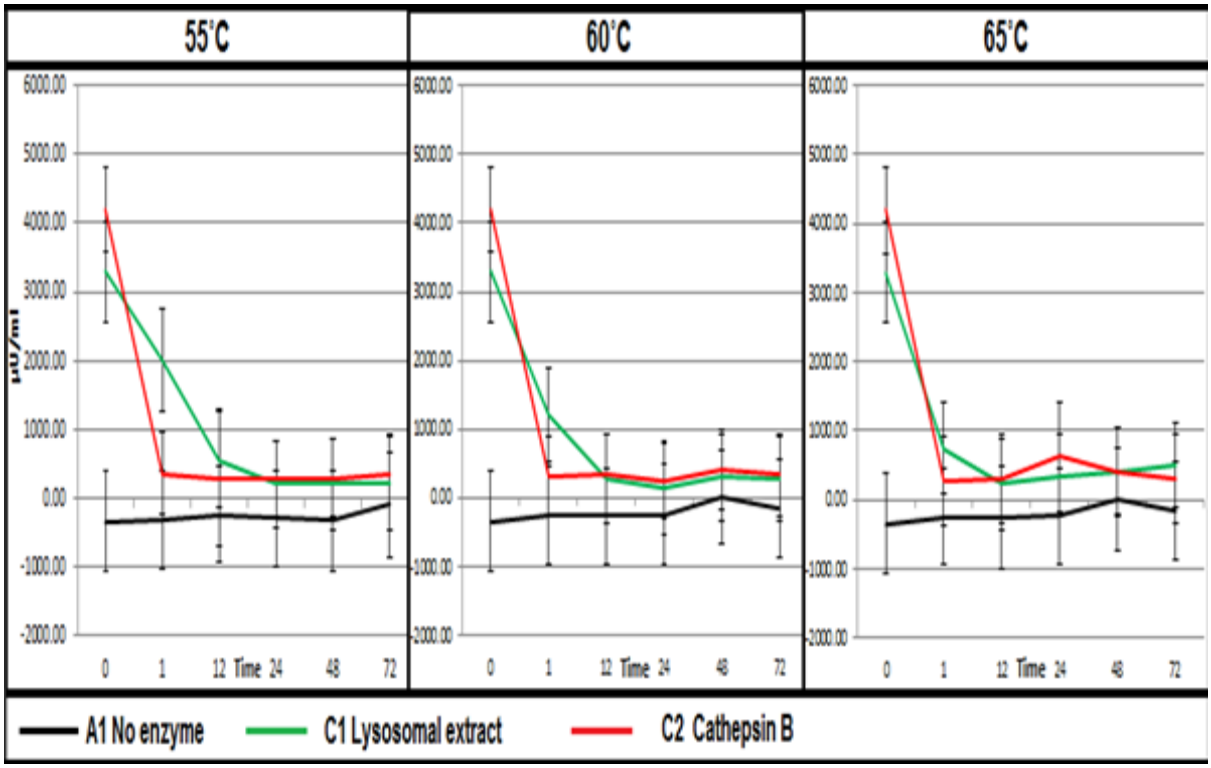


Figure 16. Comparison between conditions with and without enzyme where isolated connective tissue as substrate.

The samples containing insoluble collagen had a different development. The samples containing no enzyme (A2) had an increase in absorbance with both increasing time and temperature (Experiment A, Figure 12).

The conditions with insoluble collagen and either lysosomal extract (D1) or cathepsin B (D2) started with a higher activity than A2 at 0 hours (Figure 17).

After 1 hour the difference was significant between A2 and D1, whilst the difference between A2 and D2 was not. Across time and temperature, the only other significant difference was between A2 and D1 samples heated for 12 hours at 55°C.

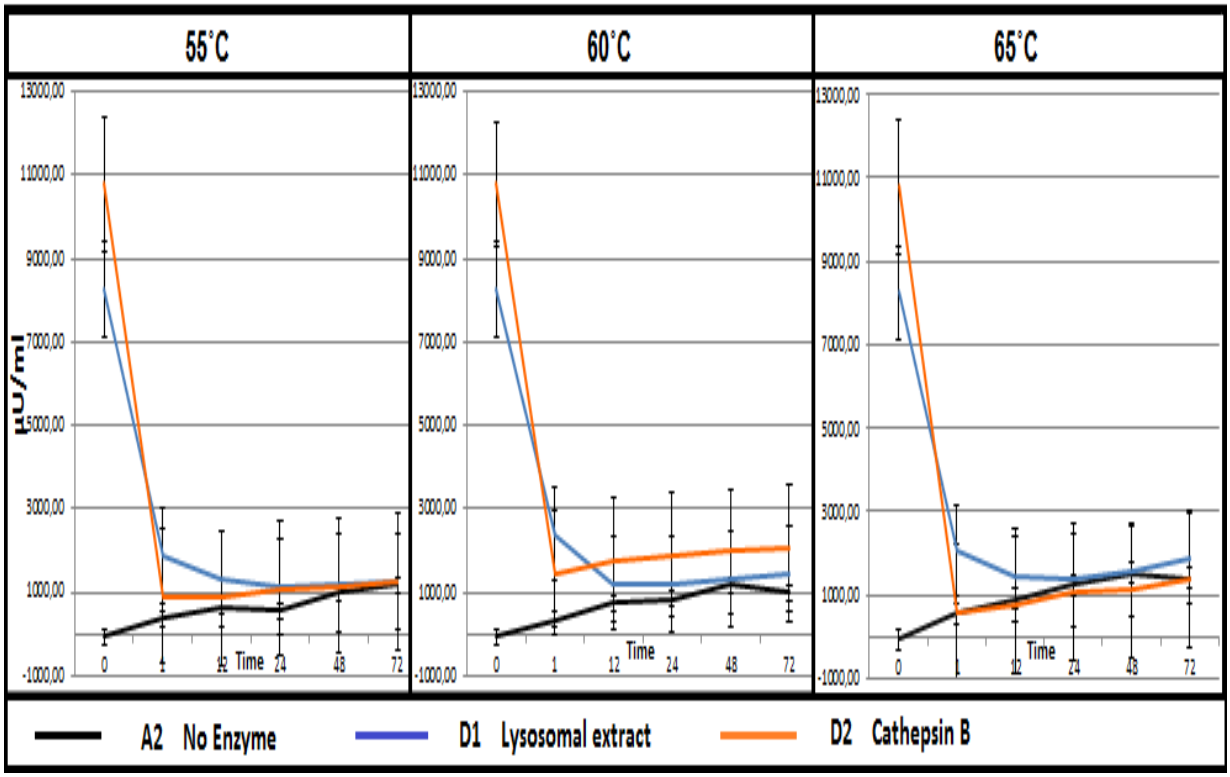


Figure 17. Comparison between the condition without and without enzyme where collagen as substrate.

To investigate if heat denatured collagen potentially measured absorbance at the same excitation and emission wavelengths as the cathepsin B substrate; a side experiment was performed. In this experiment samples containing collagen in combination with or without enzyme were picked to make a control test. The procedure followed Christensen et al. (2011), but the samples were incubated both with and without the cathepsin B substrate.

No absorbance was measured in samples incubated without substrate; however increased absorbance was again measured in A2 samples incubated with substrate.

Comparison of enzyme degradation

The first observation made when comparing the effect of temperature across time on enzymatic activity was that samples had a varying activity at 0 hours.

In Figure 18, conditions containing lysosomal extract were compared. Samples from experiment B and D started with a high activity (>8000µU/ml) compared experiment C (<4000µU/ml).

The large differences in cathepsin B activity at 0hours had little effect on samples incubated with pure cathepsin B. However for samples incubated with lysosomal extract the initial difference in cathepsin B activity continued to influence activity over time. The only exception was between samples incubated with collagen (D1) and isolated connective tissue (C1) at 55^{o}C, where D1 started with double the activity of C1 at 0 hours to almost no difference after 1 hour.

Overlooking the initial difference in cathepsin B activity, the enzyme activity follows a similar activity pattern with time and temperature independently from what substrate they were working on, though experiment D had some deviation to the pattern seen in experiment B and C.

The reduction in activity between 0 and 1 hour of heating was significant in all experiments. The significant difference seen between B1 and C1 at 0 hours continued at 1 hour for all temperatures and 12 hours at 55^{o}C and 60^{o}C. The difference between B1 and D1 was insignificant after 1 hour; however the difference became significant for all temperatures after 48hours.

In contrast with the other experiments, cathepsin B activity in lysosomal extract continued to decrease to less than 500µU/ml in B1 and C1 with increasing time. In comparison the activity in samples incubated with pure collagen (D1) levelled off above 1200 µU/ml.

Samples in experiment B measured the lowest activity for both lysosomal extract and cathepsin B, however it also had a lower initial activity compared to the other experiments.

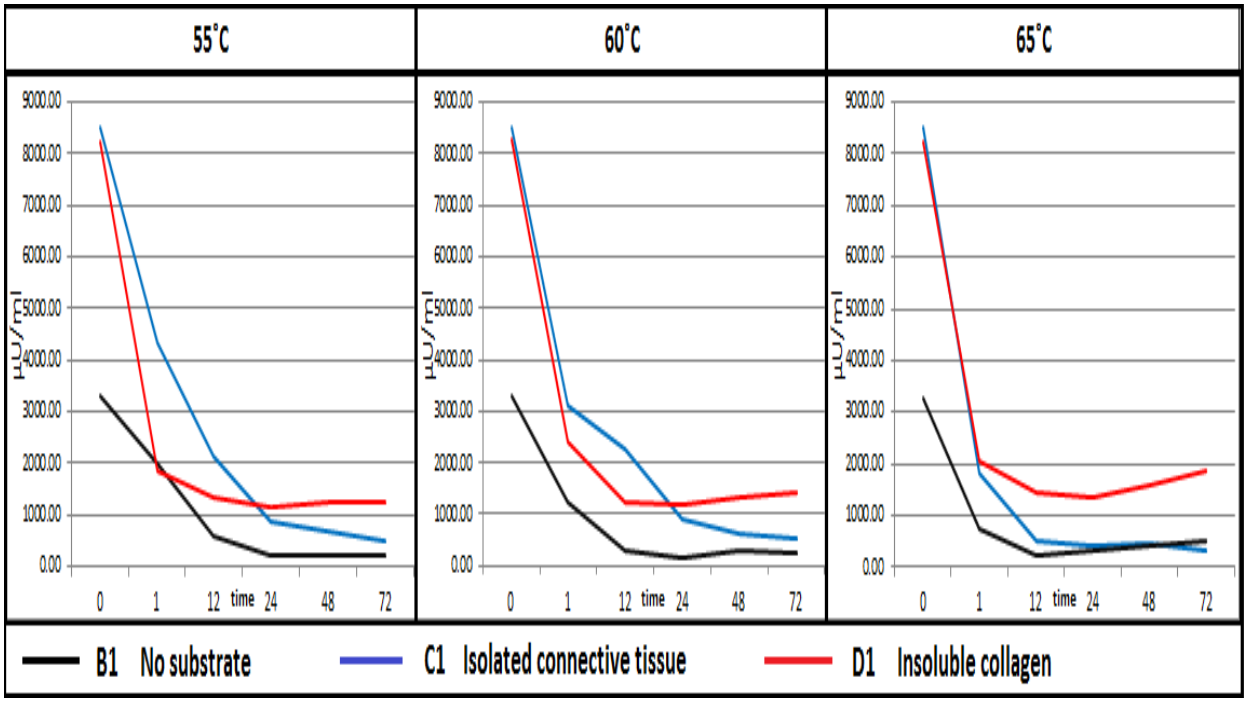


Figure 18. Comparison of cathepsin-B activity in LTLT treated samples incubated with lysosomal extract in experiment B, C and D.

Compared to lysosomal extract, Figure 19 illustrates that samples from experiment B and C had lower initial activity (<5000 µU/ml) compared with experiment D (>10000 µU/ml).

There was a rapid decrease in cathepsin B activity between 0 and 1 hour for all temperatures in samples containing pure cathepsin B. After 1 hour the activity levelled out with no significant changes with increasing time.

There was no significant difference between cathepsin B activity between the experiments LTLT treated at 55°C or 65°. At 60°C samples incubated with insoluble collagen (D2) had significantly higher activity than samples incubated without substrate (B2) at all times. D2 also had significantly higher activity than samples incubated with connective tissue (C2) LTLT treated at 60°C for 1, 24 and 72 hours.

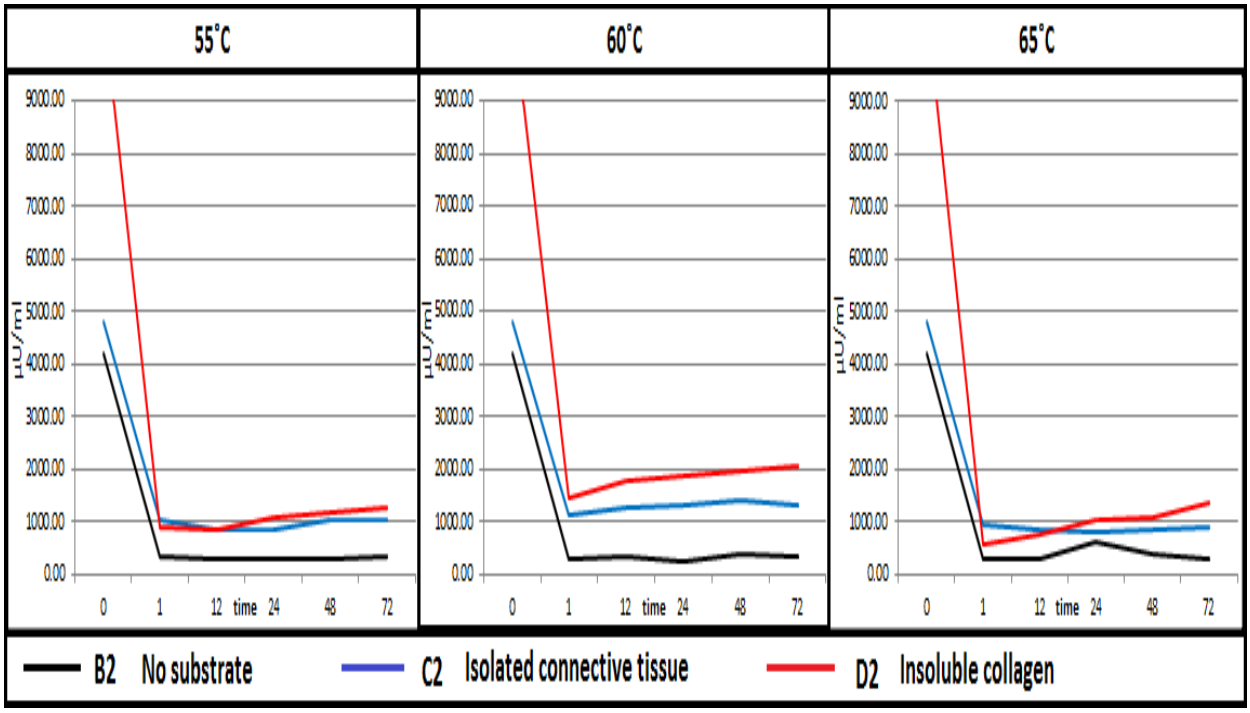


Figure 19. Comparison of cathepsin-B activity in LTLT treated samples incubated with pure Cathepsin B in experiment B, C and D.

4.2.2. Collagenase activity

Collagenase from *Clostridium histolyticum* (Sigma©) was tested in the pre experiment (3.1.4, page 20) and did not produce comparable absorbance results when combined with the *Suc-Gly-Leu-Gly-Pro-AMC* substrate. The purified lysosomal extract on the other hand showed some potential activity in two of the alerted procedures tried out, hence both of these procedures were again tried out to see if they would yield any activity.

All 1C samples were tested with an activation buffer without EDTA, with 0.8mM DTT, 0.05M $CaCl_2$, pH 5.5.Secondly all 1D samples were tested with an activation buffer without EDTA or DTT, with added 0.05M $CaCl_2$, pH 7.3. Results showed some, but very low activity (Figure 20), where neither of the two procedures resulted in any significance when compared with collagenase enzyme activity in a chosen selection of random samples containing either no enzyme or pure cathepsin B tested with the same two procedures.

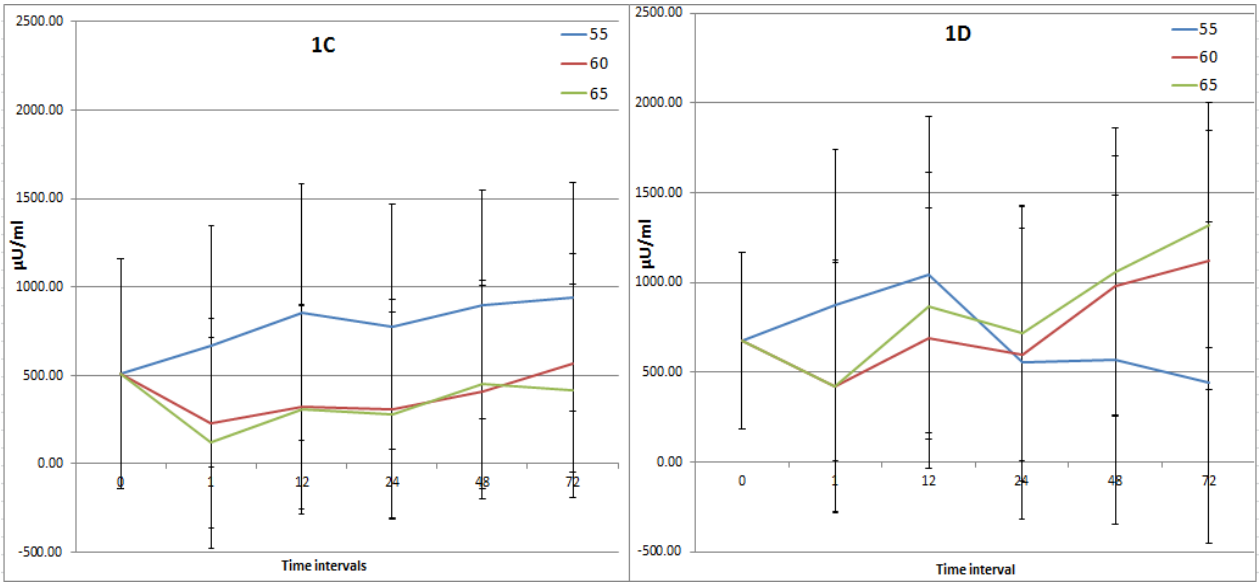


Figure 20. Collagenase activity measured in the conditions containing lysosomal extract and purified connective tissue (1C) or collagen (1D).

4.3. Visual changes observed in isolated connective tissue and collagen

In figure 21 the initial fibre structure of isolated connective tissue (1) and insoluble collagen (2) incubated without enzyme at 0hours was shown. As these were the least denatured fibres of the experiment, they were used as a baseline to compare with more denatured samples in the experiment.

The isolated connective tissue had long continuous smooth fibres, whilst the insoluble collagen had small lumps of fibre.

Texturally the isolated connective tissue, Figure 21 picture 1, was stringy with a tight woven fibrous network that was hard to pull apart in to separate clumps.

The insoluble collagen was initially freeze dried which gave it a porous texture before being mixed with the buffer, as a result the fibres easily broke apart when placing them in the tubes with the buffer.

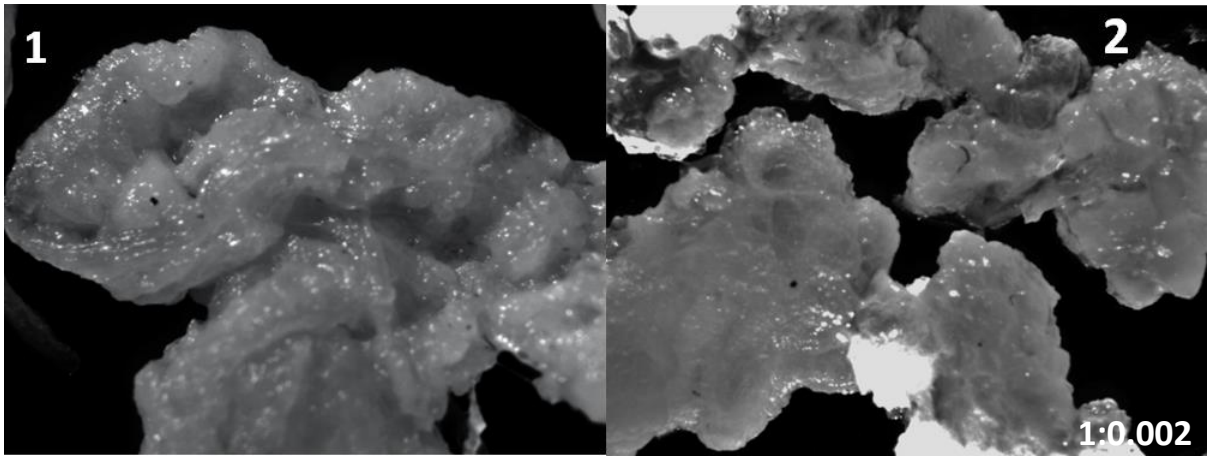


Figure21. Initial fibre structure of 1.Isolated connective tissue and 2.insoluble collagen at 0 hours magnified 32 times with a Leica MZ8 stereomicroscope.

The degradation of the two tissues from heat over time exposure was expressed visually in two main ways. One was the formation of a gel matrix in the sample liquid as the collagen in the fibres was broken down and reset as gelatine. The other was the degradation of the fibres from solid lumps that were hard to rip apart in to a softer mass that gave way when pushed on with a pipet tip.

The gel formation was more visible in the tubes containing 50mg of freeze dried insoluble collagen compared to the tubes containing purified connective tissue. However

some thickening of the sample fluid was also seen in the 72 hour conditions containing purified connective tissue, more so with increasing temperature.

After 48 hours the denaturation of the collagen was becoming apparent to the naked eye. As seen in Figure 22 the combination of collagen and enzyme, either lysosomal extract (1D) or cathepsin B (2D), resulted in more denatured collagen than seen by heat alone (2A).

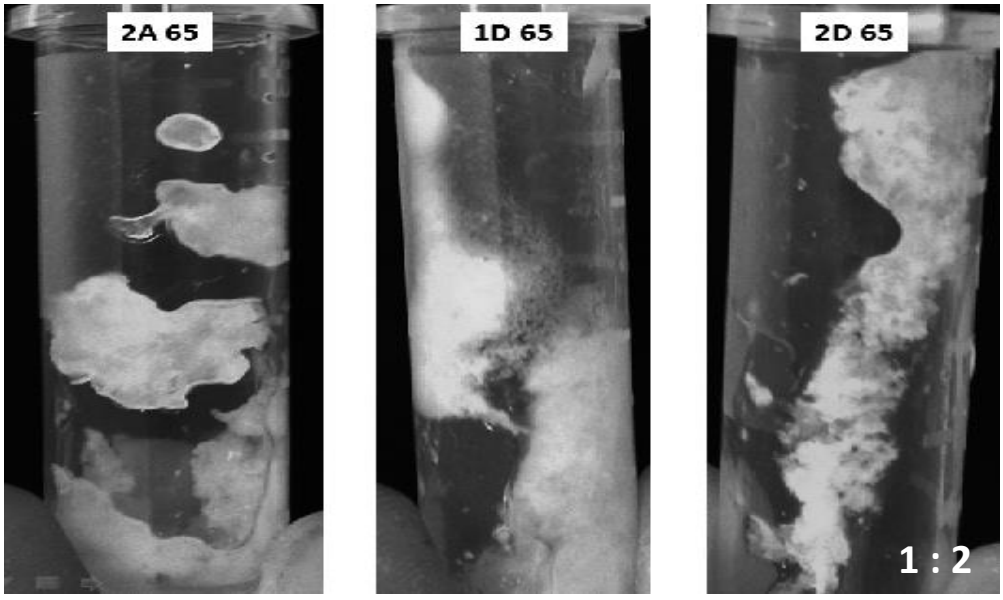


Figure 22. Initial visual observation between the denaturation of collagen after 48hours by either heat alone (2A), lysosomal extract (1D) or cathepsin B (2D).

4.3.1. Comparison of insoluble collagen samples heated at 65˚C

When comparing 65˚C samples containing collagen mixed with either no enzyme (2A), purified lysosomal extract (1D) or cathepsin B (2D) (Figure 23), it was observed that all samples were highly degraded compared to original fibres (Figure 21 picture2).

In contrast to the collagen heated with enzymes, 2A samples still contained some lumps of original structure. Whilst the samples heated with enzyme, 1D more than 2D, had completely denatured and turned in to porridge like mush with little to no recollection of its original structure.

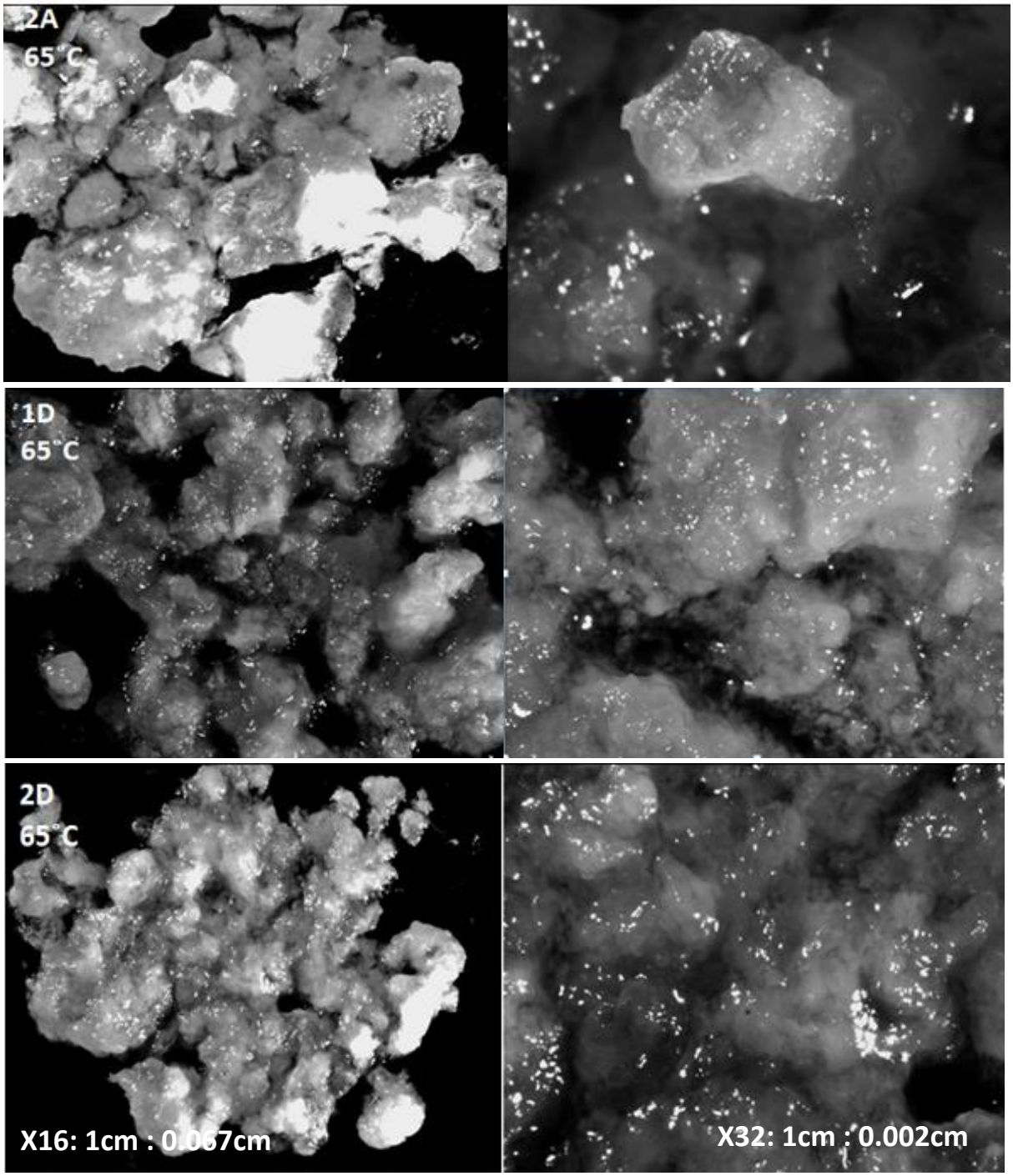


Figure 23. Magnification X16 and X32 magnification of collagen degraded with no enzyme (2A), lysosomal extract (1D), or Cathepsin B (2D) for 72 hours at 55˚C, 60˚C, and 65˚C.

4.3.2. Comparison of insoluble collagen denaturation by lysosomal extract

The largest observed denaturation was in collagen denatured by lysosomal extract.

The first change observed was that fibres had become more transparent and jelly like (Figure 24). After 72 hours the fibres readily broke apart and had porridge like texture when applying them to the optical slide fibres. The denaturation of collagen fibres were more obvious in samples cooked at 65˚C compared to 55˚C and 60˚C.

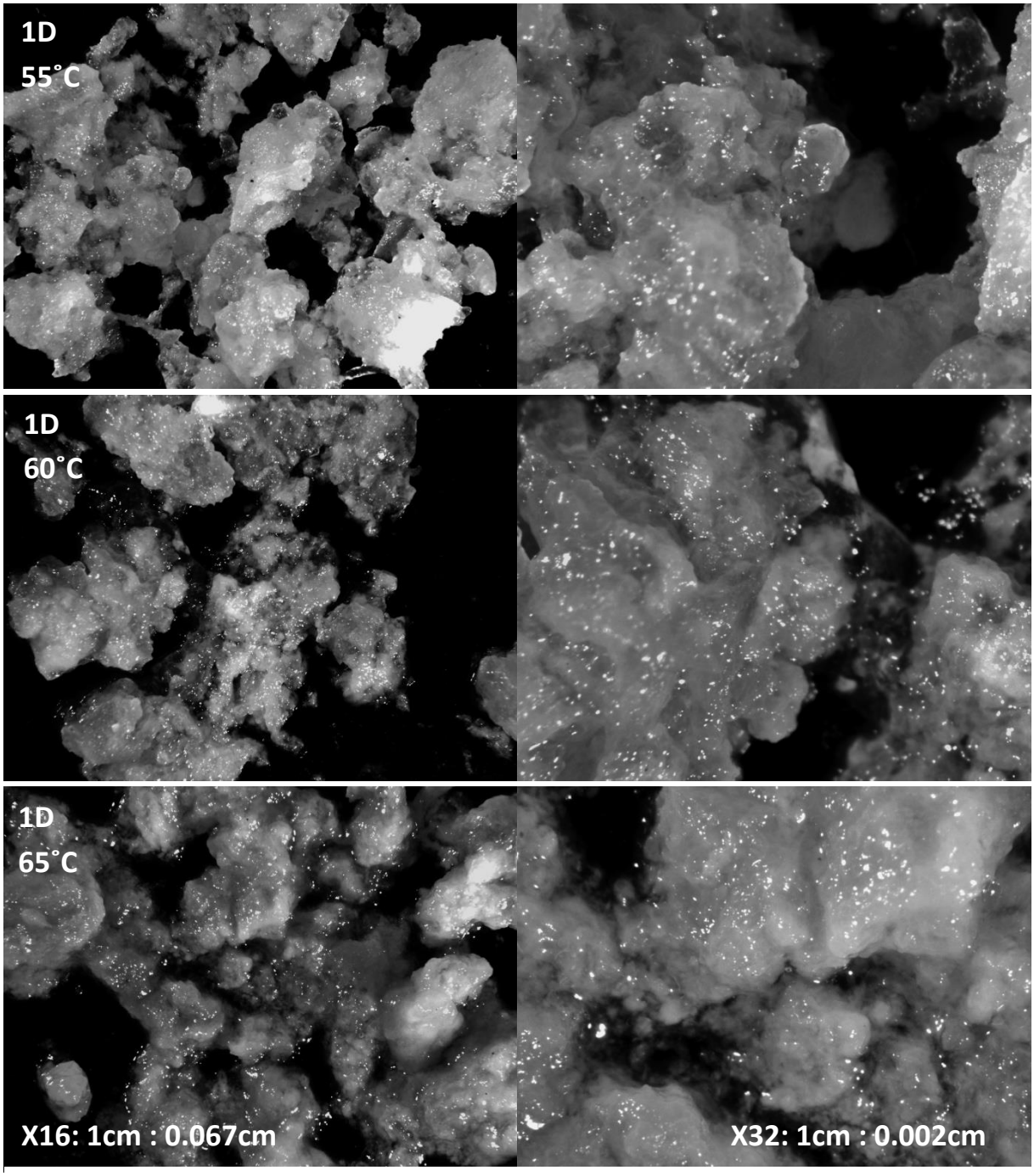


Figure 24. . Magnification X16 and X32 of Collagen from bovine achilles tendon after 72 hours magnified 16 and 32 times by Leica.

4.3.2. Comparison isolated connective tissue denaturation by lysosomal extract

After 72 hours samples containing isolated connective tissue incubated with lysosomal extract had not denatured to the jelly like porridge as collagen had.

Instead the appearance had changed from folded long white fibres tightly knitted together into transparent jagged looking lumps where single fibres were difficult to distinguish (Figure 25). Texturally the fibres still had lumps and knots; however they were soft and when pulled on by two tweezers.

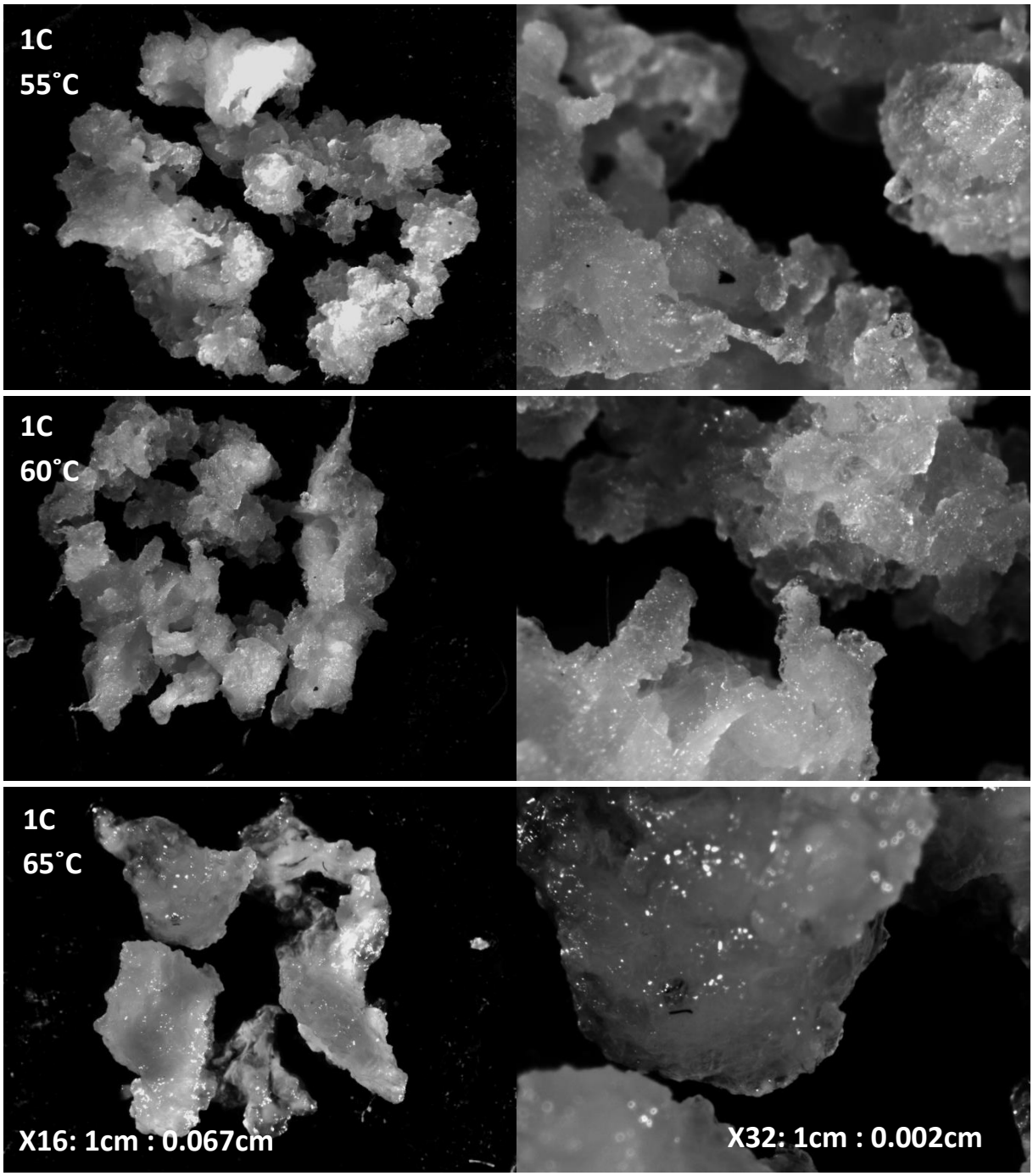


Figure 25. Magnification X16 and X32 of denatured connective tissue after 72 hours magnified 16 and 32 times by Leica.

5. Discussion

Meat tenderness is influenced by quantity of connective tissue in the muscle. In 1977 Davey and Niederer reported that temperatures above 70^{o}C was required to denature and solubilize collagen in connective tissue, however new research has found that low temperature long time (LTLT) cooking at temperatures below 70^{o}C also solubilizes collagen (Lewis and Purslow, 1989;Christensen et al. 2011).

When investigating the effect of LTLT cooking on whole muscle, this study found meat to become more tender with increasing cooking time towards 52 hours at 60˚C and 65˚C. Though similar changes were seen at both temperatures, the meat cooked at 65˚C for more than 36 hours presented the most noticeable physical alterations (Figure 10). This agrees with earlier studies investigating sensory changes in LTLT treated beef muscle (Dinardo et.al., 1984; Mortensen et.al., 2012) and studies using instrumental measurements such as Warner-Bratzler shear force, texture profile analysis and adhesion (Christensen et.al., 2011). Bouton and Harris (1981) observed a decrease in toughness when increasing cooking time from 1 to 24 hours. Although they used lower temperatures between 50˚C and 60˚C, the results in this study agreed with the findings in earlier studies as changes in tenderness was greater with increasing temperature.

Visual observations made in the present study included the shrinking of muscle bundles with time, creating gaps between individual fascicle bundles. It was further observed that perimysium changed colour and texture, and with increasing time perimysial tissue disappeared to some degree.

This was assessed as weakening of perimysial connective tissue, which was supported by the fact that the meat structure became easier to pull apart. Lewis and Purslow (1989) contributed these changes in cooked meat to the reduction of tensile strength as they saw a decrease in perimysial tissue strength at temperatures above 50˚C. Their assumptions were further strengthened by Christensen, Purslow, and Larsen (2000) which found perimysial tensile strength to decrease when measured against increasing cooking temperatures.

The observed gel structure formed in the cook loss further suggested both denaturation and solubilisation of collagen from connective tissue. Though some of the gel formation may be due to aggregation and gel formation of sarcoplasmic proteins

(Davey and Gilbert, 1974), the solidity and quantity of the gel formed in cook loss from samples LTLT treated at 65˚C for more than 48 hours indicates the presence of gelatine, hence sign of solubilized collagen.

Moving forward with the theory that collagen can be denatured and solubilized from LTLT temperatures below 70˚C; attention was directed towards proteolytic enzymes found in the sarcoplasmic proteins and their influence on collagen denaturation during LTLT cooking. Christensen (2012) confirmed that active forms of cathepsin B and L were present in cook loss form beef and pork LTLT treated at 48˚C and 63^{o}C.

To confirm or exclude the idea that these enzymes play a part in the denaturation of collagen during LTLT, this study set out to investigate the effect proteolytic lysosomal enzymes had on connective tissue in LTLT conditions.

The cathepsin B activity for both lysosomal extract and pure cathepsin B reduced rapidly at all temperatures. After 1 hour the activity was significantly lower than initial activity at 0 hours in all experiments with incubated enzyme.

Pure cathepsin B had a more rapid reduction in cathepsin B activity compared to lysosomal extract. The difference in activity can indicate that lysosomal extract contain other cathepsin like enzymes, or that the cathepsin B found in lysosomal extract was combined with a mixture of cofactors protecting them or allowing them to stay active for longer. As no research was found to confirm either of these theories, it would be interesting to investigate lysosomal extract and its enzymatic constituents in the future.

Independently of the initial activity at 0 hours, the cathepsin B activity followed a similar pattern with increasing time and temperature depending on type of enzyme, lysosomal extract or cathepsin B (Figure 13, 14 and 15). The figures show that after 1 and 12 hours the cathepsin B activity levelled out for pure cathepsin B and lysosomal extract respectively. The figures further show very little difference in enzyme activity between temperatures. The only significant difference between temperatures was observed for lysosomal extract in experiment B, which had lower activity after 1 and 12 hours of cooking at 65^{o}C compared with 55^{o}C and 60^{o}C.

This small variation between temperatures was a deviation from earlier studies where temperature has been an important variable for enzyme activity. For example when studying the influence of temperature on cathepsin B from ostrich, the optimal

temperature for cathepsin B activity was found at 50˚C (Van Jaarsveld, Naudé and Oelofsen, 1998). When raising the temperature above 50^{o}C the study experienced a rapidly decrease in activity. Ertbjeg et al. (2012) found active cathepsin B and L after 24 hours at 55˚C, whereas activity was greatly reduced after only 1 ½ hours at 70˚C. Similar results were found by Christensen et al. (2013) which recorded significantly higher cathepsin B and L activity in cook loss after 2 ½ at 53˚C and 55˚C compare with 58˚C and 63˚C. Their study further observed a significant reduction in activity after 7 ½ hours at 55˚C and 19 ½ hours at 53˚C.

The cathepsin activity of lysosomal extract measured in this study agrees with the earlier findings of cathepsin activity in cook loss, where the the activity after 1 hour was higher at 55˚C compared to 60˚C and 65˚C. This study can therefore argue that the low variance in cathepsin B activity for increasing temperature seen in this study may be the use of temperatures above 55^{o}C which was higher than in previous studies.

Cathepsin B activity had some dependence on what substrate the enzymes were incubated with, where the collagen (experiment D) had a higher activity than isolated connective tissue (experiment C) independently of time and temperature.

The difference was more prominent for lysosomal extract which had significantly higher activity with collagen for all temperatures after the 12 hour mark. Pure cathepsin B had similar tendencies, though only a few of the time points and temperatures were significantly higher with collagen compared to isolated connective tissue. There was also seen a raise in cathepsin B activity in samples incubated with collagen in experiment A.

As all experiments saw a clear difference between isolated connective tissue and pure collagen, the main differences between the two substrates were examined. The difference considered to most likely have an effect was the weight of the substrates. Connective tissue was frozen in lumps after isolation, and still contained some $CaCl_2$ from the last washing step in the procedure. The collagen used was freeze dried, hence the weight was unaffected by any other factors. It is therefore likely that there were different amount of collagen present in the tubes with 50mg isolated connective tissue and freeze dried collagen. If more collagen was available for denaturation and heat denatured collagen resulted in some unexpected interference with measured absorbance, this initial

dissimilarities in weight and amount of available collagen could explain the difference of measured activity between the substrates.

However, when preforming an enzymatic assay on the same samples without using the cathepsin B specific substrate (7-phe-Arg-amido- methyl Coumarin) no absorbance was measured at excitation and emission wavelength 355nm and 460nm respectively.

This raises a question if the pure collagen bought from Sigma© could have contained some additional cathepsin enzyme, or if there could have been some reaction between solubilized collagen and the substrate.

As no answer was found from earlier research, the cathepsin B activity measured in experiment B and C, which did not appear to have unexpected interference, was seen as better choice for representing the effect LTLT cooking had on cathepsin B activity of lysosomal extract and pure cathepsin B.

A second abnormality was seen between initial enzyme activity at 0 hours for both lysosomal extract and cathepsin B. As the same amount of stock was diluted and added to the test tubes and the dilutions were treated the same before the procedure, the difference in activity was hard to explain. The reasons proposed included inconsistencies between purifications of lysosomal extract and incomplete homogenization of the pure cathepsin B dilution.

Nevertheless the initial difference did not influence the activity pattern, and after 24 hours there was no longer a distinguishable difference indicating that time and temperature parameters had a larger influence on the enzymes cathepsin B activity than initial enzyme activity.

As the initial cathepsin B activity of lysosomal extract and cathepsin B within each experiment was close to equal, meaning the denaturing effect of the enzymes could be compared.

However, as mentioned earlier the Cathepsin B activity was higher in samples incubating lysosomal extract with collagen compared to isolated connective tissue. Therefore one could argue that the more noticeable denaturation of collagen fibres was a result of difference in enzymatic denaturation and not a difference between heat stability of the fibres.

Though the cathepsin B activity reduced to approximately the same within 1 hour for all experiment; the initial difference in cathepsin B activity between experiments was therefore considered to have little importance when comparing substrate denaturation with increasing time between experiments.

Figure 22 shows the initial observations of collagen denaturation after 48hours. The initial observation was that collagen incubated with enzyme was more denatured than samples denatured by heat alone. This initial observation was confirmed by the pictures taken with the Leica MZ8 stereomicroscope (Figure 23).The pictures illustrated a more prominent denaturation of collagen by lysosomal enzymes (D1) and cathepsin B (D2) compared with heat alone (A2) at 65˚C.

The Leica MZ8 pictures further illustrated the combined effect of thermal and enzymatic denaturation as fibres were more denatured at 65˚C compared with 55˚C (Figure 24 and 25). After 72 hours the fibres cooked at 65˚C had a transparent porridge like structure (Figure 24), whilst the samples heated at 55˚C still displayed some of the original structure qualities seen in Figure 20.

Though the isolated connective tissue did not denature in to porridge like mush, the denaturation of the connective tissue fibres became more prominent with increasing temperature (Figure 25). The observable changes in fibre colour were coupled with alterations in structural qualities such as ease to break the tissue samples apart.

This agrees with earlier observation which have found a decrease in breaking tensile strength when perimysial connective tissue was treated with temperatures above 50^{o}C (Lewis and Purslow, 1989; Christensen, Purslow and Larsen, 2000).

To confirm collagen denaturation and solubilisation future research should include methods that can quantify heat soluble and insoluble collagen such as Ringer solution (Hill, 1966) and hydroxyproline determination (Bergman and Loxley, 1963). This will give better overview of the LTLT induced collagen denaturation than observations made with a stereomicroscope alone.

The difference observed in in denaturation of pure collagen and isolated connective tissue may be due to a difference in the tissue quaternary structure. However no information was obtain on post mortem age of the animals the substrates were acquired from. The study was therefore unable to comment on the possible influence of heat

stable intramolecular crosslinks which would affect total unaltered fraction during denaturation of collagen (Powell, Hunt and Dikeman, 2000). It could therefore be beneficial to investigate the effect of LTLT cooking on denaturation of the three different collagen fractions.

Collagenase activity could not be confirmed due to defective methodology in the enzymatic assay procedure, however it was confirmed that the lysosomal extract was capable of degrading the collagenase specific substrate. Collagenase is a heat sensitive molecule, where collagenase derived from fish has been seen to denature at temperatures below 40^{o}C (Park et al, 2002). Hence the fact that the collagenase purchased from Sigma© was derived from *Clostridium histolyticum* and not from an animal source may have been the reason why no results were seen in the dilution series. The influence of collagenase activity in lysosomal extract during denaturation is an interesting topic to consider for future research. However other methodologies should be considered, including methods that have been proven successful in earlier research such as “determination of proteinase activity by gelatine digestion”.

6. Conclusion

The present study investigated the effect of low temperature long time (LTLT) cooking on proteolytic enzyme activity and their effect on bovine connective tissue and collagen.

LTLT cooking of whole bovine trapezius muscle at 60°C and 65°C for cooking times up to 52 hours resulted in improved tenderness with increasing time and temperature. The increased tenderness was coupled with an observable change in perimysial connective tissue. With increasing cooking time the connective tissue became more brittle and the fascicles were easier to tear apart.

As visible connective tissue decreased and the cook loss had an increasing amount of gelatine, the study concluded that collagen found in the connective tissue must have been denatured and solubilized to form gelatine.

Based on earlier studies proteolytic activity was suspected to play a role in the observed denaturation. The study therefore continued with an *in vitro* study to assess cathepsin B enzymatic activity of across LTLT treated pure cathepsin B and lysosomal extract. The enzymes were also coupled with pure bovine collagen and isolated connective tissue to monitor the denaturation process.

Cathepsin B activity decreased rapidly with increasing time and temperature for both enzymes investigated. The activity decreased more rapidly in pure cathepsin B compared to lysosomal extract, with little influence from what temperature they were heated at.

Lysosomal extract was still noticeably active after 1 hour and for some conditions at 12 hours. The difference in reduction rate of activity was thought to come from active cofactors in the lysosomal extract supporting cathepsin B activity. However as this is not confirmed in this study or by earlier research, the effect of cofactors on proteolytic lysosomal enzymes could be an interesting topic for future investigations.

Collagen or connective tissue incubated with enzyme was more denatured than the substrate incubated with buffer alone. The denaturation of collagen incubated with lysosomal extract was the most prominent, where the collagen structure was broken down to a mush without any solid structure. In contrast to collagen the structure of

connective tissue did not collapse to create a mush; instead the structure became more brittle and was easier to break apart by pulling on the fibres.

Hough little change was seen in enzyme activity after 24 hours the incubated structures and the LTLT treated whole muscle both had great change to their physical characteristics after this time point. This may be due to a combined effect of thermal and enzymatic denaturation, and would be interesting to research further.

Even though the study saw a relatively rapid decrease in cathepsin B enzyme activity during LTLT cooking above 55^{o}C for 1 to 72 hours, it also found that the connective tissue and collagen incubated with enzyme was more denatured than tissue denatured by heat alone.

To get further understanding of the proteolytic effect of lysosomal enzymes during LTLT cooking, future studies should address a broader variety of enzymes and possible cofactors, and their influence on meat structure components.

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Appendix 1.

Statistical data, significance between time and temperature per conditions:

Experiment A

A1		0H	55C					60C					65C				
		0C	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
	0H																
55C	1H	1															
	12H	1	1														
	24H	1	1	1													
	48H	1	1	1	1												
	72H	1	1	1	1	1											
60C	1H	1	1	1	1	1	1										
	12H	1	1	1	1	1	1	1									
	24H	1	1	1	1	1	1	1	1								
	48H	1	1	1	1	1	1	1	1	1							
	72H	1	1	1	1	1	1	1	1	1	1						
65C	1H	1	1	1	1	1	1	1	1	1	1	1					
	12H	1	1	1	1	1	1	1	1	1	1	1	1				
	24H	1	1	1	1	1	1	1	1	1	1	1	1	1			
	48H	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
	72H	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	

2A		0C	55C					60C					65C				
		0H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
0C	0H																
55C	1H	1															
	12H	0,09	1														
	24H	0,32	1	1													
	48H	0,0001	0,3722	1	1												
	72H	0,0001	0,0155	0,7931	0,4092	1											
60C	1H	1	1	1	1	0,2427	0,0073										
	12H	0,0147	1	1	1	1	1	0,9921									
	24H	0,002	1	1	1	1	1	0,8339	1								
	48H	0,0001	0,0137	0,771	0,3845	1	1	0,0064	0,9817	1							
	72H	0,0001	0,3144	1	1	1	1	0,1987	1	1	1						
65C	1H	0,3144	1	1	1	1	0,4092	1	1	1	0,3845	1					
	12H	0,0004	0,7479	1	1	1	1	0,5943	1	1	1	1	1				
	24H	0,0001	0,0028	0,4605	0,1537	1	1	0,0013	1	1	1	1	0,1537	1			
	48H	0,0001	0,0001	0,0038	0,0005	0,736	0,4092	0,0001	0,0298	0,1677	1	0,7931	0,0005	0,3604	1		
	72H	0,0001	0,0002	0,0375	0,006	1	1	0,0001	0,1907	0,5808	1	1	0,006	1	1	1	

Experiment B

1B		0C	55C					60C					65C				
		0H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
0C	0H																
55C	1H	0,0001															
	12H	0,0001	0,0001														
	24H	0,0001	0,0001	0,0001													
	48H	0,0001	0,0001	0,0001	1												
	72H	0,0001	0,0001	0,0001	1	1											
60C	1H	0,0001	0,0001	0,0006	0,0001	0,0001	0,0001										
	12H	0,0001	0,0001	1	0,0001	0,0001	0,0001	0,0119									
	24H	0,0001	0,0001	0,0001	1	1	1	0,0001	0,0001								
	48H	0,0001	0,0001	0,0001	1	1	1	0,0001	0,0001	1							
	72H	0,0001	0,0001	0,0001	1	1	1	0,0001	0,0001	1	1						
65C	1H	0,0001	0,0001	1	0,0001	0,0001	0,0001	0,0001	1	0,0004	0,0001	0,0001					
	12H	0,0001	0,0001	0,0001	1	1	1	0,0001	0,0001	1	1	1	0,0001				
	24H	0,0001	0,0001	0,0001	1	1	1	0,0001	0,0001	1	1	1	0,0001	1			
	48H	0,0001	0,0001	0,0001	1	1	1	0,0001	0,0001	1	1	1	0,0001	1	1		
	72H	0,0001	0,0001	0,0001	1	1	1	0,0001	0,0001	1	1	1	0,0001	1	1	1	

2B		0C	55C					60C					65C				
		0H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
0C	0H																
55C	1H	0,0001															
	12H	0,0001	1														
	24H	0,0001	1	1													
	48H	0,0001	1	1	1												
	72H	0,0001	1	1	1	1											
60C	1H	0,0001	1	1	1	1	1										
	12H	0,0001	1	1	1	1	1	1									
	24H	0,0001	1	1	1	1	1	1	1								
	48H	0,0001	1	1	1	1	1	1	1	1							
	72H	0,0001	1	1	1	1	1	1	1	1	1						
65C	1H	0,0001	1	1	1	1	1	1	1	1	1	1					
	12H	0,0001	1	1	1	1	1	1	1	1	1	1	1				
	24H	0,0001	1	1	1	1	1	1	1	1	1	1	1	1			
	48H	0,0001	1	1	1	1	1	1	1	1	1	1	1	1	1		
	72H	0,0001	1	1	1	1	1	1	1	1	1	1	1	1	1	1	

Experiment C

1C		0C	55C					60C					65C				
		0H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
0C	0H																
55C	1H	0,0001															
	12H	0,0001	0,0001														
	24H	0,0001	0,0001	1													
	48H	0,0001	0,0001	1	1												
	72H	0,0001	0,0001	1	1	1											
60C	1H	0,0001	0,0001	0,5586	0,0005	0,0005	0,0007										
	12H	0,0001	0,0001	0,7073	1	1	1	0,0001									
	24H	0,0001	0,0001	0,2016	1	1	1	0,0001	1								
	48H	0,0001	0,0001	1	1	1	1	0,0001	1	1							
	72H	0,0001	0,0001	0,6813	1	1	1	0,0001	1	1	1						
65C	1H	0,0001	0,0001	1	1	1	1	0,6276	0,6413	0,1619	0,7569	0,6139					
	12H	0,0001	0,0001	0,4757	1	1	1	0,0001	1	1	1	1	0,4093				
	24H	0,0001	0,0001	1	1	1	1	0,0003	1	1	1	1	1	1			
	48H	0,0001	0,0001	1	1	1	1	0,0015	1	1	1	1	1	1	1		
	72H	0,0001	0,0001	1	1	1	1	0,013	1	1	1	1	1	1	1	1	

2C		0C	55C					60C					65C				
		0H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
0C	0H																
55C	1H	0,0001															
	12H	0,0001	0,0001														
	24H	0,0001	0,0001	1													
	48H	0,0001	0,0001	1	1												
	72H	0,0001	0,0001	1	1	1											
60C	1H	0,0001	0,0001	1	1	1	1										
	12H	0,0001	0,0001	1	1	1	1	1									
	24H	0,0001	0,0001	1	1	1	1	1	1								
	48H	0,0001	0,0001	1	1	1	1	1	1	1							
	72H	0,0001	0,0001	1	1	1	1	1	1	1	1						
65C	1H	0,0001	0,0001	1	1	1	1	1	1	1	1	1					
	12H	0,0001	0,0001	1	1	1	1	1	1	1	1	1	1				
	24H	0,0001	0,0001	1	1	1	1	1	1	1	1	1	1	1			
	48H	0,0001	0,0001	1	1	1	1	1	1	1	1	1	1	1	1		
	72H	0,0001	0,0001	1	1	1	1	1	1	1	1	1	1	1	1	1	

Experiment D

1D		0C	55C					60C					65C				
		0H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
0C	0H																
55C	1H	0,0001															
	12H	0,0001	0,0001														
	24H	0,0001	0,0001	1													
	48H	0,0001	0,0001	1	1												
	72H	0,0001	0,0001	1	1	1											
60C	1H	0,0001	1	0,0002	0,0001	0,0001	0,0001										
	12H	0,0001	0,0715	1	1	1	1	0,0001									
	24H	0,0001	0,0375	1	1	1	1	0,0001	1								
	48H	0,0001	1	1	1	1	1	0,0012	1	1							
	72H	0,0001	1	1	1	1	1	0,0004	1	1	1						
65C	1H	0,0001	1	1	0,6736	1	1	0,3256	0,6607	0,5001	1	1					
	12H	0,0001	0,0236	1	1	1	1	0,0001	1	1	1	1	0,3966				
	24H	0,0001	0,0023	1	1	1	1	0,0001	1	1	1	1	0,0919	1			
	48H	0,0001	0,1606	1	1	1	1	0,0001	1	1	1	1	1	1	1		
	72H	0,0001	1	1	1	1	1	0,01	1	1	1	1	1	1	1	1	

2D		0C	55C					60C					65C				
		0H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H	1H	12H	24H	48H	72H
0C	0H																
55C	1H	0,0001															
	12H	0,0001	0,0001														
	24H	0,0001	0,0001	1													
	48H	0,0001	0,0001	1	1												
	72H	0,0001	0,0001	1	1	1											
60C	1H	0,0001	1	1	1	1	1										
	12H	0,0001	1	1	1	1	1	0,0001									
	24H	0,0001	1	0,7931	1	1	1	1	1								
	48H	0,0001	0,6078	0,4092	1	1	1	0,4605	1	1							
	72H	0,0001	0,1696	0,0848	1	1	1	0,1031	1	1	1						
65C	1H	0,0001	1	1	1	1	0,5943	1	0,6607	0,1606	0,0354	0,0032					
	12H	0,0001	1	1	1	1	1	1	1	1	0,6607	0,2004	1				
	24H	0,0001	1	1	1	1	1	1	1	1	1	1	1	1			
	48H	0,0001	1	1	1	1	1	0,0071	1	1	1	1	0,6211	1	1		
	72H	0,0001	0,2243	0,1169	1	1	1	0,1406	1	1	1	1	0,0049	0,2621	1	1	