Tensile Properties of Ovine Skeletal Muscle Fibres: Thermal Denaturation and Selective Removal of Titin.

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Abstract

The stiffness of chemically skinned single ovine skeletal *psoas major* muscle fibres is affected by many post-mortem events. These same events produce highly variable eating quality attributes in whole meat.

This thesis focuses on three aspects of these events; sarcomere length, titin degradation and heat. Each of these factors was experimentally manipulated *in vitro* and the effect on the stiffness of single muscle fibres was measured. A technique was developed to apply strain to the fibre while recording the corresponding stress output.

To study the effect of sarcomere length on single fibre stiffness before and after heating to 80°C (cooking), each fibre was set to one of six sarcomere lengths; 1.6, 1.8, 2.1, 2.7, 3.0, and 4.2 μm. Oscillations of 5 Hz were designed to stretch each fibre by 5 nm/half sarcomere. At sarcomere lengths below resting length (2.1 μm), the stiffness of uncooked fibres remained relatively unchanged, but appeared to increase in cooked fibres in proportion to the sarcomere length prior to cooking.

The effect of selective titin degradation on the structural characteristics of single fibres, before and after cooking, was also examined using a low concentration of trypsin to selectively digest titin. The stiffness of raw fibres was unaffected by titin removal, but heating to 80°C caused significant changes in the stiffness of trypsin-treated fibres.

Two fixed sarcomere lengths were subsequently used to demonstrate the temperature dependence of fibre stiffness; 1.6μm and 2.1μm. Although the fibre stiffness response at both sarcomere lengths was similar during heating, when the temperature was reduced from 80°C the response of fibres with a sarcomere length of 2.1 μm

exhibited lower stiffness responses to each temperature than fibres with a sarcomere length of 1.6 μm.

The length of the I-band appears to be important, especially after cooking. As shown in Chapter 2, the length of the I-band contributed to the final stiffness of the thermally denatured fibres, independent of the number of cross-bridges that had formed prior to cooking. As the I-band increased in length in the raw state, the more compliant the fibres became after cooking.

This thesis provides information on, and a better understanding of, the structural and mechanical properties of single muscle fibres as a result of three different post-mortem factors. Understanding how these factors affect the meat on a cellular level can help us to better control the environmental and postmortem influences on whole meat quality.

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Chapter 1

General Introduction

1.1 Foreword

Meat is an important component in the diet of the developed world and to maintain consumer appeal must match other foodstuffs on the supermarket shelf for quality, consistency and value. There is also intense competition between different sources of animal proteins: sheep and beef meats, the major animal products of New Zealand, must compete with the highly efficient and intensive production systems for pork, poultry and fish. The key component to ensuring a financially successful meat industry in New Zealand is to ensure that the appearance and eating quality of its meat products have the highest possible consumer appeal.

Taste, tenderness, odour, colour and juiciness have been adjudged by consumers to be the most important sensory factors, in order of importance (Issanchou 1996). Undoubtedly, ensuring that meat is tender is a key requirement for an acceptable eating experience. Tenderness has proved to be a highly variable attribute of meat, particularly beef, and defining production and processing systems to produce meat of a consistent tenderness, in a cost effective manner, has been recognised around the world as a high priority for the meat industry (Morgan *et al.*, 1991).

The actual process by which meat becomes tender appears to involve the postmortem proteolytic degradation of structural proteins in muscle, but the details of this process are not well understood. A number of protease enzymes have been implicated in this process, particularly the calpain enzyme system (Tsuji and Imahori, 1981; Koohmaraie *et al.*, 1988), but also the lysosomal cathepsin proteases

(Eino and Stanley, 1973; Ouali *et al.*, 1984), and significant effort has been devoted to describing the behaviour of these enzymes in meat. However, equally important to understanding the process of proteolytic tenderisation are the interactions between the protease systems and the structural proteins that they degrade. It is unclear which structures within meat/muscle need to be degraded and, moreover, what role these proteins may play in the problem of variability in tenderness in meat.

This study considers the role of key structural proteins and their responses to changes in sarcomere length, proteolytic degradation and temperature. It is important to keep in mind that this work demonstrates how the mechanical properties of single skeletal muscle fibres are affected by the factors listed above. However, this thesis does not make a direct comparison of whole meat tenderness and single fibre stiffness, but seeks to provide evidence that a potential link exists through the literature reviewed and the data submitted. To investigate the effect of sarcomere length, proteolytic degradation and temperature on the stiffness of ovine skeletal muscle fibres, a method of measuring the mechanical integrity of individual, isolated muscle fibres was employed.

1.2 The Structure of Muscle

1.2.1 Hierarchical Organisation of Muscle Tissue

Skeletal muscle is a multi-component tissue that attaches indirectly to bone via tendons. Different types of skeletal muscle perform different functions, but all have the same basic structure. Skeletal muscles are constructed in varying proportions from skeletal muscle cells or fibres, connective tissue, blood vessels and peripheral nerves. Muscles are generally compartmentalised by connective tissue which imparts certain mechanical properties as well as providing passive structural support.

Whole muscles are covered in a protective connective tissue sheath called the epimysium. The muscle is divided into bundles of muscle fibres (fasciae) which are also surrounded by connective tissue, the perimysium. Further compartmentalisation results whereby each muscle fibre is surrounded by yet another layer of connective tissue termed the endomysium. These muscle fibres are mainly composed of hundreds or thousands of myofibrils, the cylindrical and highly ordered structures which house the thick and thin myofilaments; the protein assemblies responsible for the sliding action associated with muscle contraction.

1.2.2 The Sarcomere

In the early 1950's both A.F. Huxley and Niedergerke (1954) and H.E. Huxley and Hanson (1954) independently suggested the sliding filament theory as the mechanism by which skeletal muscle contracts to produce force. Three years later A.F. Huxley clarified the structural basis of contraction by postulating the existence of an active site on the actin filament together with a sliding member of an elastic nature sited on the myosin filament (Huxley 1957).

The primary contractile unit of the muscle cell is the myofibril comprising a complex but highly ordered lattice of thick and thin filaments organised into repeating structural units called sarcomeres (Figure 1.1). Myosin is the principal component of the thick filaments centered within each sarcomere, and conformational changes associated with its specific actin-activated and Ca^{2+}-stimulated adenosine triphosphatase (ATPase) activity produce muscle contraction by the sliding of adjacent thick and thin filaments (Huxley and Hansen, 1954; Huxley and Niedergerke, 1954). Each myosin molecule is composed of two heavy chains, two regulatory chains and two alkali light chains, and each thick filament

contains 288 myosin molecules in a bipolar arrangement (Skubiszak and Kowalczyk, 2002). The myosin tails form the thick filament, with globular heads projecting at regular helical intervals towards either end of the filament to contact binding sites on filamentous actin of the thin filaments. The thin filaments contain several proteins, of which the most abundant is actin. Actin is made up of many globular units (G-actin) joined to form a filamentous chain (F-actin). Two of these chains form a double helix about 1 μm in length; and this forms the backbone structure of the thin filament. A double stranded α-helical chain of tropomyosin runs the length of each F-actin filament, the primary function of which is to produce steric blocking of the myosin binding sites present on each actin monomer. It is the calcium dependent regulation of this steric blocking that is responsible for activation of skeletal muscle contraction. This regulation is provided by troponin. Troponin is a complex of three protein sub-units located at intervals of 385Å along the thin filament; Troponin I (TnI), Troponin C (TnC) and Troponin T (TnT). Each troponin complex, by way of tropomyosin, regulates seven actin monomers. TnI binds the troponin complex to the actin; TnC binds Ca^{2+}, and TnT binds the troponin complex to the tropomyosin. As Ca^{2+} levels increase it cooperatively binds to TnC initiating a conformational change within the troponin complex, the TnI releases from the F-actin and the attached tropomyosin no longer sterically blocks actin-myosin binding sites (Stryer 1995). This allows the myosin to attach to the binding site on the actin monomer forming an actomyosin complex termed a cross-bridge. In most muscle, full cross-bridge overlap occurs at a sarcomere length of ≤2.4 μm (Huxley 1974; Cooke and Franks, 1980). In frog muscle for example, the thick filament has been measured at 1.65 μm in length, and the thin filament at 1.05 μm. Sarcomere lengths of less than 1.65 μm will result in damage due to thick filament contact with the Z-

line (Huxley 1957; 1974). At sarcomere lengths of 2.0 µm or less, the thin filaments overlap and can cause some disruption in the region of the M-line (Huxley 1974).

The I-band is the length of the thin filament where no cross-bridges are formed whereas the A-band is formed by myosin and any overlapping thin filaments. The A-band is comprised of a slightly less dense region termed the H-band and in the centre of this H-band is the M-line. The function of the M-line is to anchor the thick filaments by way of several myosin binding proteins. The massive structural protein, titin, attaches to the myofilaments to hold them in place within the sarcomere and provide some elasticity to the muscle cell (Figure 1.1).

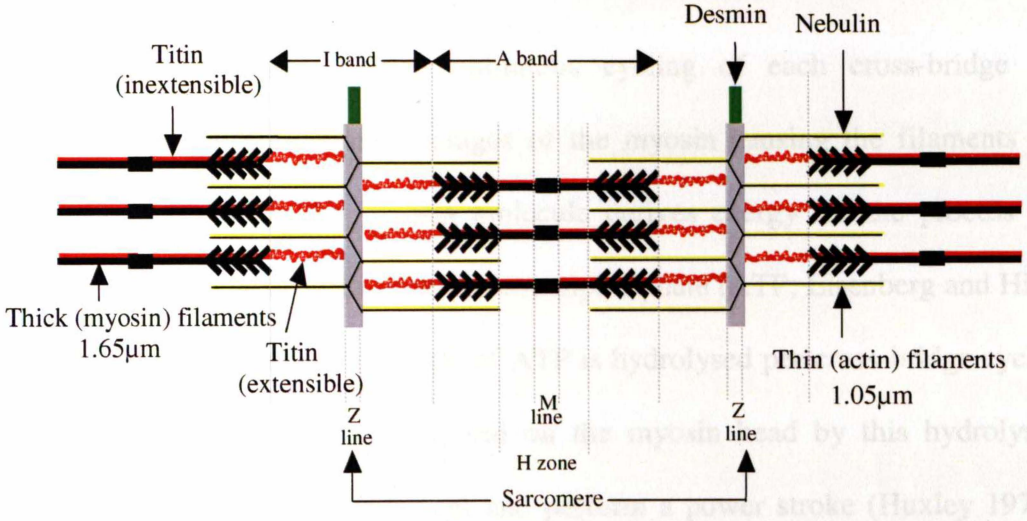

Figure 1.1 A diagram of a contractile unit, showing banding patterns, thin and thick filaments, and the areas of overlap that form cross-bridges (adapted from Huxley 1957; Hedrick 1994).

Nebulin can be found running along the length of the actin filament and is thought to act as a template for thin filament assembly during myofibrillogenesis and in mature muscle may act as a scaffold for thin filament stability (Robson *et al.*, 1991). Titin is the primary structural protein involved in anchoring the thick filaments to the Z-lines at either ends of the sarcomere. An inextensible region of tightly bound titin

spans the length of the thick filament with an extensible section bridging the I-band gap between the thick filament and the Z-line (Linke *et al.*, 1996). This holds the myosin filament in position, especially when the thick and thin A-band filaments are dislocated during stretch (Locker 1987).

Desmin is another structural protein found running perpendicular to the cell axis, its function being to bind the Z-lines of adjacent myofibrils together, thus keeping all sarcomeres aligned with their respective excitatory structures (t-tubules and sarcoplasmic reticulum).

1.3 Biochemistry of Contraction and Rigor

Contraction is produced by the continuous cycling of each cross-bridge in conjunction with conformational changes of the myosin causing the filaments to slide past one another. The myosin molecule derives energy for the process of contraction from the hydrolysis of adenosine triphosphate (ATP; Eisenberg and Hill, 1978). It is thought that one molecule of ATP is hydrolysed per cross-bridge cycle, and the conformational change imposed on the myosin head by this hydrolysis causes it to attach to the thin filament and perform a power stroke (Huxley 1974; Eisenberg and Hill, 1978). This action, repeated many times by multiple myosin heads in a population of muscle fibres results in contraction (Huxley 1974). Smooth muscle contraction relies on the spatial mismatch between helical spacings of the myosin heads on the thick filaments and the helical spacing of the F-actin filaments (refer Figure 1.2; Eisenberg and Hill, 1978).

1.3.1 Ca^{2+}-Induced Myosin Conformational Changes

Striated muscle contraction is instigated by the depolarisation of the sarcolemma and t-tubules by an action potential initiated from the motorneurons. This causes an increase in Ca^{2+} concentration in the myofibrillar sarcoplasm from the sarcoplasmic reticulum (SR), the cellular Ca^{2+} reservoir (Nakajima and Endo, 1973). The t-tubule system is an invagination of the sarcolemmal membrane and projects into each single muscle cell, surrounding myofibrils. The invaginations lie between the lateral cisternae of the SR. TnC sub-units on the thin filaments bind Ca^{2+}, exposing the active binding sites on actin. This allows the attachment and sliding of thick and thin filaments, both of which remain at a constant length (refer Figure 1.2). The duration of muscle contraction is defined by the continuation of depolarizing action potentials. If the available ATP is depleted before contraction is complete actomyosin cross-bridges are unable to detach. Skeletal muscle also has the ability to contract to varying degrees and this is achieved by motor unit summation. Motor units are defined as a single motor neuron and all the muscle fibres it directly innervates, summation is the number of motor units being stimulated at any given moment.

When the action potentials stop, the sarcolemmal membrane re-polarises and ATP is used to actively transport Ca^{2+} out of the myofibrillar sarcoplasm and back into the sarcoplasmic reticulum. The removal of Ca^{2+} reverses the conformational change of the troponin complex, sterically blocking the active binding site on the actin monomer and inhibiting contraction. ATP continues to be hydrolysed by the myosin ATPase, but the concealed binding sites do not facilitate attachment. As a result no movement is produced and thick and thin filaments return to their resting positions and relaxation occurs. Relaxation occurs as a result of decreasing Ca^{2+} concentration, and muscle cells remain relaxed while Ca^{2+} levels are low, and ATP is readily available.

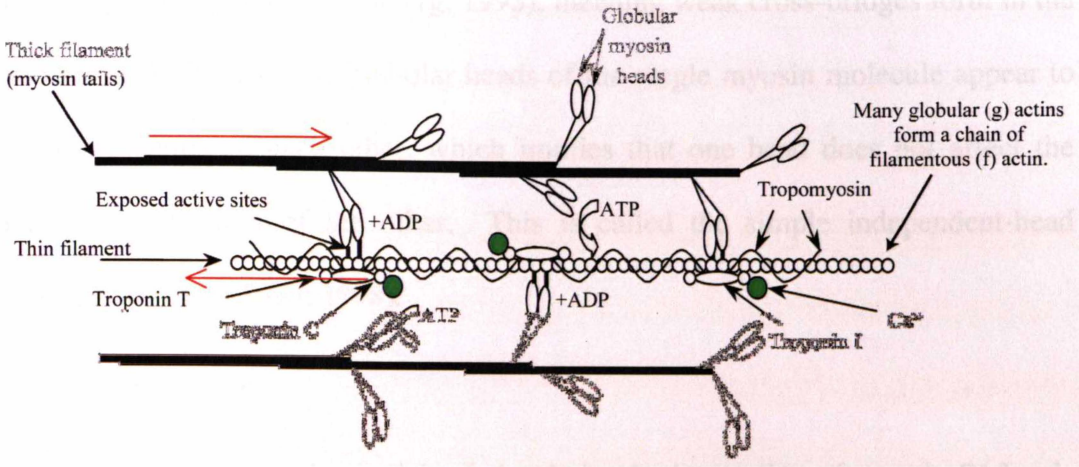

Figure 1.2 Active muscle contraction (red arrows depict the direction of movement).

1.3.2 Myosin ATPase

Myosin is an ATPase, and its physical properties are changed in the presence of ATP (Engelhardt and Lyubimova, 1939). Actin and myosin will actively form cross-bridges in the absence of ATP, and this complex can only be broken by its presence (Huxley 1957). ATP dissociates the actomyosin complex and, during active contraction, the attachment of ATP to each myosin head precedes the next power stroke (refer Figure 1.3). During contraction, the intracellular calcium concentration increases and the ATPase activity of actin-activated myosin increases by approximately 50-100 times (Bárány 1967).

1.3.3 Weak and Strong Cross-bridges

Weak Cross- bridges

When muscle cells are in a relaxed state, myosin ATPase activity is greatly reduced by tropomyosin, however, actomyosin interaction still occurs, albeit weak and transitory in nature (Cooke 1986). These weakly bound cross-bridges are characterised by the flexibility and mobility of the attached myosin head (Schoenberg 1993; 1998). They form during non-contractile cycling in the relaxed

state (Jung *et al.*, 1989; Schoenberg, 1993), meaning weak cross-bridges form in the presence of ATP. The two globular heads of the single myosin molecule appear to act independently of each other, which implies that one head does not affect the attachment behaviour of the other. This is called the simple independent-head model (Schoenberg 1993; 1998).

Strong Cross-bridges

Strong cross-bridges can be explained simply by the interaction of myosin S1 heads and actin to form bonds that keep the myosin heads attached, oriented and immobile. Unlike weakly bound cross-bridges, the attachment of the second head is dependent on the position and orientation of the first head. This is termed the double-headed cross-bridge model (Schoenberg 1993; 1998).

During muscle contraction, each cross-bridge cycle involves a conformational change of the myosin S1 and S2 sub-units in order to produce the power 'stroke' (refer Figure 1.3; Huxley and Simmons, 1971; Cooke 1997; Tyska and Warshaw, 2002). To maintain continuous cycling attachment of the actomyosin cross-bridges, only a small proportion of the myosin heads bind to active sites at any given time. This is controlled primarily by the position of the active actin binding sites relative to the myosin heads. Approximately 20% of the actin binding sites are situated correctly for cross-bridge formation at any given time during active contraction (Hedrick *et al.*, 1994).

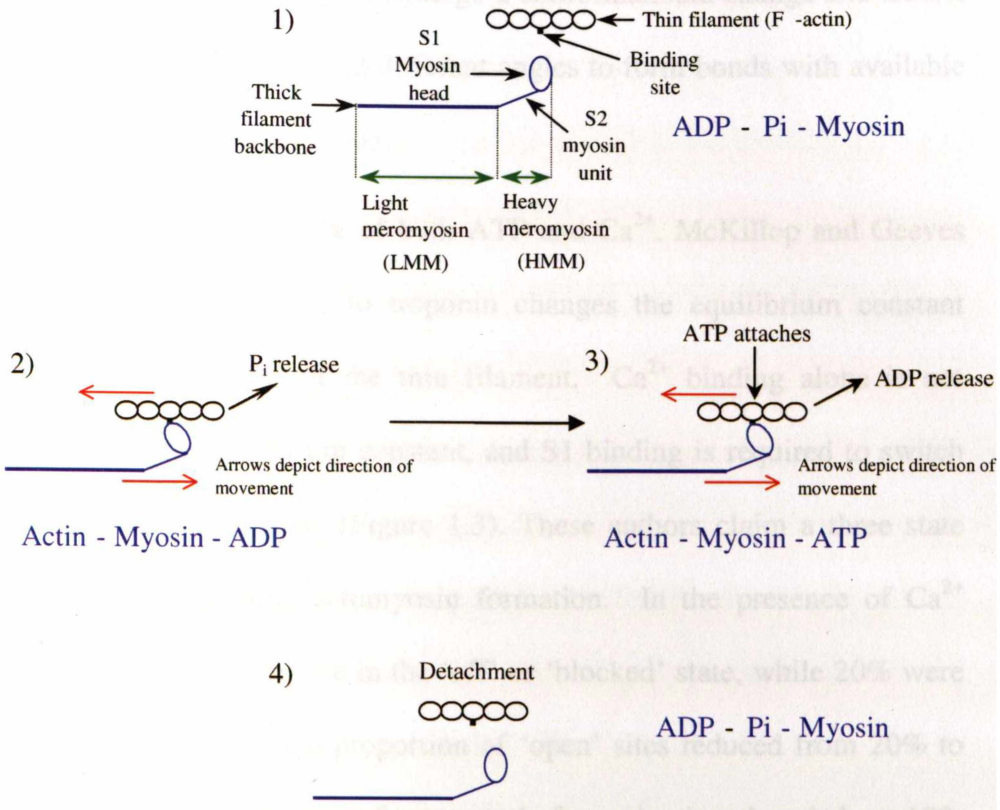

Figure 1.3 Proposed mechanism for the actin myosin contractile 'power stroke'

1.3.4 Rigor Bonds

When the level of ATP drops from its normal physiological level of ~5 mM to below 0.1 mM, myosin and actin cease to slide past each other and interact to form strong rigor bonds. One example of this is the mass formation of cross-bridges post-mortem, termed rigor mortis (rigor). This involves the interaction of all myosin heads within reach of adjacent thin filaments (Cooke and Franks, 1980; Higuchi *et al.*, 1995).

Rigor cross-bridge formation should not be confused with the actively cycling cross-bridges of muscle contraction (Yamamoto and Herzig, 1978). The latter occur in the presence of both ATP and Ca^{2+}, whereas rigor forms in the absence of ATP. In

comparison, rigor cross-bridges do not undergo a conformational change and almost all myosin heads end up distributed at different angles to form bonds with available actin binding sites (Yamada *et al.*, 2003).

Rigor can also occur in the absence of both ATP and Ca^{2+}. McKillop and Geeves (1993) state that calcium binding to troponin changes the equilibrium constant between the on and off states of the thin filament. Ca^{2+} binding alone is not sufficient to change this equilibrium constant, and S1 binding is required to switch the thin filament into the on state (Figure 1.3). These authors claim a three state equilibrium model is regulating actomyosin formation. In the presence of Ca^{2+} approximately 80% of actin sites are in the 'off' or 'blocked' state, while 20% were 'open'. In the absence of Ca^{2+} the proportion of 'open' sites reduced from 20% to $\leq$2%. In the absence of Ca^{2+}, the rate of actomyosin formation is reduced about 100-fold but strong binding of one myosin molecule can cooperatively accelerate the binding of neighboring myosin heads (Kad *et al.*, 2005). Troponin/Tropomyosin regulatory units have been shown to dramatically reduce the frequency of actomyosin formation in low concentrations of Ca^{2+}. Thermal fluctuations of tropomyosin can lead to the exposure of the low proportion of actin binding sites in the absence of Ca^{2+} (Kad *et al.*, 2005).

1.4 Meat Tenderness

Tenderness is a complex attribute of meat that is influenced by a wide range of factors. Historically, the collagen content of muscle has received considerable attention, and remains important in two respects. Firstly, differences in the eating quality of different muscles can be attributed to collagen content and characteristics (Dutson 1974; Light and Bailey, 1983). Secondly, the effect of animal age on the

eating quality of muscles is also associated with changes in collagen (Dutson 1974; Horgan *et al.*, 1991). Although the total content of collagen can affect eating quality, it is often the solubility of collagen that has been found to provide the better predictor of tenderness. Extensive cross linking of the collagen fibrils, a process that increases with age, decreases the solubility of collagen during cooking and is associated with increased toughness (Young and Braggins, 1993).

However, while collagen can provide some explanation for tenderness under certain conditions, two further important contributors are the state of contraction of the muscle, and the proteolytic enzymes responsible for the degradation of muscle structure. Both, but particularly the former, are affected by events that occur during the development of the state of rigor mortis in muscle.

1.4.1 Postmortem Energy Metabolism

Permanent muscle stiffness develops at the onset of rigor mortis and is a result of formation of an actomyosin complex. This complex cannot be dissociated due to a lack of ATP (Partmann 1963). In the physiological state, ATP is kept in constant supply in aerobic conditions by the oxidative phosphorylation of, primarily, carbohydrate and fatty acid substrates. When the muscle is rendered ischemic at slaughter, ATP resynthesis depends on glycolysis of muscle glycogen stores and regeneration by the creatine phosphokinase and myokinase reactions. The accumulation of anaerobic metabolites primarily, lactate, gradually inhibits glycolytic enzymes, ATP levels decline, and rigor onset begins (Bechtel and Best, 1985).

ATP is essential for cell survival and is found in muscle cells primarily bound to magnesium – Mg-ATP. It is in the Mg-ATP state that it can be hydrolysed by

myosin to control binding to actin, by the sarcoplasmic reticulum Ca^{2+}-ATPase to control free myoplasmic Ca^{2+} concentrations, and by sarcolemmal Na^+/K^+-ATPase to maintain membrane polarisation. Although many different ATPase systems exist, Bendall (1975) and Hamm (1977) suggested that the total ATPase activity post-mortem is comprised primarily of myosin ATPase.

In the early postmortem period, creatine phosphokinase provides an important mechanism for the resynthesis of ATP, using creatine phosphate (CP) as the substrate. This reaction is near equilibrium, so that any changes in the concentration of the reactants will produce a very rapid re-establishment of the ratios of reactants and products. This means that, in general, ATP levels remain fairly constant until ~70% of the creatine phosphate has been degraded (Bechtel 1986).

$$\text{CrP} + \text{MgADP} \xleftrightarrow[\text{Phosphokinase}]{\text{Creatine}} \text{MgATP} + \text{Cr} \qquad \text{Cr} = \text{Creatine}$$

Figure 1.4 Resynthesis of ATP from creatine phosphate (CrP)

ATP hydrolysis, followed by its resynthesis by creatine phosphokinase produces inorganic phosphate (P_i) as a metabolite. P_i stimulates the regulatory enzymes of glycolysis and oxidative phosphorylation contributes to increased glycolysis. It therefore acts together with decreasing ATP and increasing ADP, to enhance ATP resynthesis through glycolysis as the CP concentrations become depleted (Walsh *et al.*, 2002).

The metabolites of anaerobic glycolysis are lactate, H^+ and NAD^+. Lactate accumulates from the reduction of pyruvate by lactate dehydrogenase (LDH), while simultaneously oxidising the NADH cofactor to NAD^+ (Figure 1.5).

$$\text{Pyruvate} + \text{NADH} \overset{\text{LDH}}{\leftrightarrow} \text{Lactate} + \text{NAD}^+ + \text{H}^+$$

Figure 1.5 Conversion of Pyruvate to Lactic Acid

NADH is produced by glycolysis by the glyceraldehyde 3-phosphate dehydrogenase step (G3PDH). NADH would, in oxygenated tissue, be oxidised to NAD^+ in mitochondria, but this is prevented by the anoxic state of postmortem muscle. NAD^+ is a necessary substrate for G3PDH, without which glycolysis will cease. Therefore, in anaerobic conditions, the LDH reaction acts as a substitute for NAD^+ production. The gradual accumulation of lactate eventually inhibits this reaction, and glycolysis ceases due to the depletion of NAD^+.

A further ATP resynthesis reaction is stimulated by the decline in ATP and the accumulation of ADP. ATP can be resynthesised by the adenylate kinase (myokinase) reaction and because this too is an equilibrium reaction and would be inhibited by the accumulation of adenosine monophosphate (AMP), a further reaction, the deaminase reaction, removes AMP and helps to keep the myokinase reaction active:

$$2\ \text{ADP} \longleftrightarrow \text{ATP} + \text{AMP}$$

Figure 1.6 The adenylate kinase (myokinase) reaction

$$\text{AMP} \xrightarrow{\text{Deaminase}} \text{IMP} + \text{NH}_3$$

Figure 1.7 The Deaminase Reaction

Inosine monophosphate (IMP) is then further degraded to inosine and hypoxanthine (Lawrie 1998).

Despite the combined effects of the myokinase and deaminase reactions, the ATP concentration eventually falls below 100 μM, cross-bridges bind irreversibly, and rigor mortis develops.

1.4.2 Postmortem Contractile Events

Two forms of contractile events have been identified in postmortem muscle, and are referred to as rigor shortening (or heat shortening), and cold shortening. Rigor shortening is an active post-mortem process that results in rigor mortis.

Rigor Shortening

At a pH of below 6.3, a temperature >20°C, and an ATP concentration of <2.5μmol/g muscle, whole muscles, or muscle strips, maintained in the ischemic and anoxic conditions found postmortem, become less extensible and, when maintained in isometric conditions, begin to contract. This phenomenon is more pronounced at higher temperatures (Honikel *et al.*, 1983), and as a result is often referred to as heat shortening.

One possible explanation for the contraction is an increase in intracellular calcium (Ca^{2+}) Honikel *et al.*(1983) proposed the possibility of a pH dependent sarcoplasmic reticular uptake system, which has a pH optimum of 6.3 but this systems ability to sequester Ca^{2+} decreases rapidly when the pH falls below this. However, although measurements of intracellular Ca^{2+} at these pHs have not been carried out in skeletal muscle, measurements in cardiac muscle suggest that there is an increase in intracellular Ca^{2+} levels as the pH declines (Fabiato and Fabiato, 1978). An alternative explanation for rigor shortening relates to changes in calcium sensitivity in conditions of low ATP concentrations. Bailey (1984) demonstrated a marked increase in sensitivity to Ca^{2+} when ATP concentrations fell below 100 μM. This

mechanism would explain not only the increasing contracture at relatively low Ca^{2+} concentrations, but also why the contracture is associated with the later phases of the rigor process.

Cold Shortening

Contracture of postmortem muscle also develops in response to reduced temperature. The effect can be detected at temperatures as high as 25°C, but becomes increasingly severe as the temperature is reduced to 0°C, where shortening of up to 60% can occur (Davey and Gilbert, 1974). Cold shortening is distinct from rigor shortening because the extent of the contracture in response to temperature decreases as the pH declines (Chrystall and Devine, 1978). At a pH of around 7, when ATP concentrations are near normal, the response to cold is most marked, but the response reduces in a linear relation with decreasing pH and disappears when the pH reaches 6.

Locker and Hagyard (1963), Davey and Gilbert (1974), Buege and Marsh (1975) and Cornforth *et al.*(1980) all agree that a rise in Ca^{2+} level initiated by the low temperatures is responsible for cold contraction and resultant shortening. Jeacocke (1982) demonstrated that the efflux of Ca^{2+} from single intact fibres preloaded with the calcium isotope, ^{45}Ca, increased when the temperature of the fibre was reduced.

In 1974, Davey and Gilbert postulated that the postmortem rise in intracellular Ca^{2+} was due to the effects of temperature on the SR membrane. It was thought that chilled temperatures lowered the efficiency of the ATP-dependent ionic pump and also caused an increase in the efflux of Ca^{2+} from the SR. The phospholipids in membranes undergo very distinct temperature-dependent phase transitions that increase porosity and change the electrical properties (Davey and Gilbert, 1974).

An alternative proposal is that the increase in Ca^{2+} is associated with mitochondria. Mitochondria take up Ca^{2+} with low affinity but high capacity, and contribute to the control of intracellular Ca^{2+} (Cornforth *et al.*, 1980). Buege and Marsh (1975) suggested that cold shortening was a result of anoxia-induced Ca^{2+} release from muscle mitochondria because cold shortening in beef and lamb is most pronounced in red muscles which contain high numbers of mitochondria. However, Bendall (1975) pointed out that the link between the concentrations of mitochondria and the severity of cold shortening is not invariant, since the m. *longissimus dorsi* of pigs, a muscle consisting predominantly of white fibres and containing low mitochondria, shortens vigorously in response to cold.

The disappearance of the cold shortening response in muscle with low pH has not been explicitly explained. High H^+ concentrations reduce the sensitivity of the contractile apparatus to Ca^{2+}, and, together with increased P_i and low [ATP], reduce the ability to generate force. Together, these effects may explain the low level of responsiveness to temperature under conditions of low pH in postmortem muscle.

1.4.3 Shortening and Toughness

Postmortem muscle shortening to any degree is impossible to avoid, as it is a natural result of the 'running-down' of the biochemical processes within a muscle, though its extent can be controlled to a certain degree by control of temperature at which the muscle enters rigor. The control of contracture in postmortem muscle is important to meat because of its impact on tenderness. Locker and Hagyard (1963) were the first to demonstrate that cold shortening toughens meat, a phenomenon that has since been amply demonstrated (Partmann 1963; McRae *et al.*, 1971; Bouton and Harris, 1972; Bouton *et al.*, 1973; Rowe 1977). Toughness increases with increasing

shortening until approximately 40% shortening, at which point meat is extremely tough. Interestingly, with still further shortening, meat becomes more tender again; an effect attributed to internal damage to the meat by super-contraction of the sarcomeres (Davey and Gilbert, 1974).

The effect of sarcomere length on meat toughness appears to relate closely to the extent of overlap between thick and thin filaments (Marsh and Carse, 1974). This effect can be demonstrated by stretching muscle pre-rigor, so that post-rigor sarcomere lengths are increased; under these conditions, the meat is made more tender (Vada-Kovacs 1996). The reason for the importance of sarcomere length on tenderness has not been fully explained, but relates to changes that occur in meat during cooking. The importance of cooking is inferred from measurements of resistance to shear in uncooked meat. Cold shortened meat in the uncooked state is actually more tender than normal, (Purchas 1973; Davey and Gilbert, 1975b; Dransfield and Rhodes, 1976), and the toughening expected of shortened meat only becomes apparent once the myofibrillar proteins are denatured by temperatures greater than 55°C. Evidently, the structural characteristics of the denatured actomyosin strands produced by cooking are sensitive to the extent of overlap between thick and thin filaments.

1.4.4 Role of Proteolytic Events in Meat Tenderness

Although the degree of shortening in meat will have a significant impact on tenderness, it is the case that, at rigor, meat with sarcomeres of resting length is not of a tenderness that would appeal to most consumers. Further tenderisation is needed, and this depends on the proteolytic degradation of muscle structure. Davey and Gilbert (1967) referred to these structural changes as aging.

Aging occurs at storage temperatures above freezing (Davey and Gilbert, 1967). The structural alterations that occur during aging are most evident in the Z-line region of the sarcomere, where many of the structural and regulatory proteins are found. These include the primary structural protein, titin (Locker 1987). The A-band or contractile component consists of both thin and thick filaments, but proteolysis does not appear to have much effect on these filaments or the cross-bridges that have formed during rigor on-set (Davey and Gilbert, 1967). However, some weakening is evident within the components of the thin filament (Davey and Dickson, 1970).

Early work by Davey and Dickson (1970) divided the aging process into three phases. The first involves the disassociation of the myofibrils at their attachment junctions. The second is the loss of the thin filament anchoring site near the Z-line, and the final phase is the dissolution of the Z-line resulting in an almost complete loss of structure. The decrease in measured shear force (increase in tenderness), reflects the separation of the filaments involved with the I-band from the N-line anchor sites (Davey and Dickson, 1970). This process is progressive and occurs over a long period of time. Nishimura *et al.* (1998) indicated that the myofibrillar component of whole muscle aging is fairly rapid until about 10 days postmortem, thereafter it tapers off and the process of aging slows down. However, there is still some variation between muscles (Penny 1980).

In contrast to Davey and Dickson (1970), Nishimura *et al.* (1998) suggested that tenderness occurs as a two-step process. The first is a rapid phase involving the proteolytic breakdown of myofibrils. The second stage involves a slower structural weakening of the sarcolemma and connective tissue. Although this work identified changes in the tensile characteristics of the connective tissue, more generally the

importance of tenderness to changes in the connective tissue in meat has been difficult to establish convincingly (Davey 1983).

Not all proteins within a muscle cell are sensitive to proteolytic attack during the early stages of storage and, as a result, have very little contribution to the increase in tenderness during the aging period. Those most likely to have the greatest effect on overall tenderness have been identified using shear force analysis and comparative protein gel electrophoresis (Huff-Lonergan *et al.*, 1995). Many variations of the basic electrophoretic technique exist, but in this case the most popular is sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), combined with identification of specific proteins and their breakdown products using immunoblotting techniques.

The particular proteins whose postmortem degradation primarily contributes to increased tenderness after aging have not been clearly identified. It is known that thick and thin filaments are not proteolytically degraded during storage however, it seems reasonable to assume that other structural proteins in muscle will have an important role to play in the toughness of meat. The main structural proteins apart from the thick and thin filaments are titin, nebulin, desmin and possibly troponin T, although this last protein is usually considered a regulatory rather than structural protein. Each of these proteins has been identified as being proteolysed during aging and therefore, all are potential candidates for involvement in the tenderisation process.

Titin

Titin (also known as connectin) is termed a 'gap filament' and is the largest protein found in muscle, with a molecular weight of approximately 3,000,000 Dalton (Da) (Locker 1984; Trinick 1992). It is approximately 1 μm in length, and runs from the

Z-line to the M-line. The primary function of titin in a mature muscle cell is to hold the thick filaments in register and central in the sarcomere, and provide structural support and continuity, especially during stretch (refer Figure 1.8). It is thought that, in a developing cell, the titin forms a template for thick filament assembly (Trinick 1992).

Titin spans both the I-band and the A-band, but performs fundamentally different functions within each of these positions. The A-band region of titin appears to be joined to the thick filament by way of C protein, and is thought to be inextensible and rigid. On the other hand, the titin that spans the I-band has a specific region of extensibility which enables the muscle fibre to be stretched a considerable distance while still maintaining myofibrillar structure, and allowing the filaments to reposition in the correct place upon release of the stretch (Linke *et al.*, 1996).

During storage, titin appears to form an electropheretic doublet as it degrades from titin 1 (T1) to titin 2 (T2). T2 has a molecular weight of approximately 2,400,000 Da (Huff-Lonergan *et al.*, 1996a). The intensity of this protein band increases from day 3 postmortem and is still present in small amounts after 28 days (Ho *et al.*, 1996). A third degradation product is also evident at approximately 1 day postmortem and has an approximate molecular weight of 1,200,000 Da (Matsuura *et al.*, 1991; Huff-Lonergan *et al.*, 1996). Huff-Lonergan *et al.* (1995) concluded that titin is degraded at a faster rate in tender beef compared to tough beef and discussed the observed quantities of titin with regard to 'tough' and 'tender' meat, as measured by tenderness analysis and sensory panels. These authors also drew the conclusion that degradation of titin is related to postmortem tenderisation, but there is no indication as to whether this degradation is causative or a by-product of separate proteolytic events causing structural changes.

Nebulin

Nebulin is an 800,000 Da protein (Trinick 1992) connected to, and running the length of, the thin filament, with its terminal end attached in the Z-line. It is the second largest protein in the muscle cell and is also regarded by Locker (1984) as a 'gap filament'. It too is about 1 μm in length and its function also appears to be structural. It is thought that it anchors the thin filament to the Z-line and, like titin, it forms a template during muscle development that aids in exact filament assembly (Trinick 1992; Huff-Lonergan *et al.*, 1996b).

Unlike titin, nebulin does not appear as an electrophoretic doublet during degradation. The intensity of the single nebulin band will decrease after about 3 days postmortem and is generally gone by day 7 (Ho *et al.*, 1996). The degradation of nebulin was also correlated to the increase in tenderness (Huff-Lonergan *et al.*, 1995).

Although it has not yet been shown, it appears likely that titin and/or nebulin have pivotal roles in the postmortem increase in tenderness. Their size, structural properties, location, and interactions with other key proteins in the cell makes them likely candidates.

Desmin

Desmin is a structural protein with a molecular weight of 53,000 Da (Bárány *et al.*, 1995). It forms filaments of 10 nm lengths, the diameters of which are half way between actin (6 nm) and myosin (15 nm), giving desmin the name 'intermediate filament'. Desmin filaments appear to hold adjacent myofibrils together by forming a "...network of linked collars..." around the Z-lines (Young *et al.*, 1980). Once desmin begins to degrade, the Z-lines of adjacent myofibrils separate, altering the

structural integrity of the whole muscle. According to Ho *et al.* (1996), desmin intensity has significantly decreased after 3 days postmortem and gone by day 7.

Troponin T

Troponin T (TnT) is one of three sub-units of troponin (Figure 1.2). The degradation products of TnI (24,000 Da; Bárány *et al.*, 1995), and TnC (18,000 Da; Bárány *et al.*, 1995), have not been correlated with the increase in postmortem tenderness. TnT (37,000 Da; Bárány *et al.*, 1995), on the other hand, has been shown to degrade in parallel with a decrease in whole meat toughness (Penny and Dransfield, 1979; Uytterhaegen *et al.*, 1992). The disappearance of TnT correlates with the appearance of a 30,000 Da protein band, thought to be a primary breakdown product of TnT (Uytterhaegen *et al.*, 1992).

TnT is responsible for binding the whole troponin unit to tropomyosin, which is attached to the thin filament. Penny and Dransfield (1979) postulated that with this important structural responsibility, the loss of TnT would result in the troponin multi-unit dissociating from the thin filament and hence the potential for textural change.

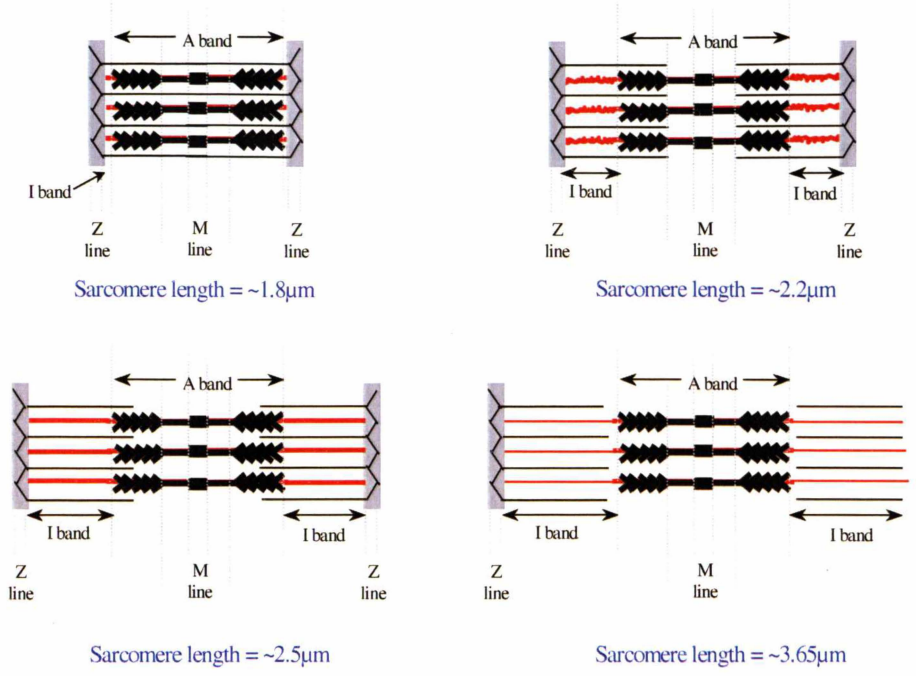

Figure 1.8 The expansion of the I-band and the subsequent decrease in cross-bridges (Adapted from Huxley 1974).

1.4.5 Proteolytic Enzymes Associated with Tenderisation

Two principle proteolytic systems have been identified as playing a role in the postmortem tenderisation of meat: the calpain system and the lysosomal cathepsins. The current view is that the calpains are the predominant proteases involved postmortem.

Calpain System

Calpain is a calcium-activated neutral protease (CANP; Ouali *et al.*, 1984). The entire Calpain system exists in the form of two isoenzymes; m-calpain, μ-calpain., and a third inhibitory polypeptide referred to as Calpastatin (Goll *et al.*, 2003). The differences between each isoenzyme relate to their calcium sensitivity. μ-calpain is activated by the presence of μM concentrations of Ca^{2+}, and m-calpain requires mM concentrations. Each isoenzyme is a heterodimer composed of 80,000 Da and 30,000 Da sub-units (Tsuji and Imahori, 1981). It is thought that m-calpain has little

to do with meat tenderisation as it undergoes complete inactivation due to autolysis (Koohmaraie 1996), however, μ-calpain retains some of its activity and thus is the only isoenzyme involved in the process of tenderisation (Koohmaraie 1996). Kasuga and Umazume (1990) showed that the threshold concentration of Ca^{2+} needed for degradation was as low as 0.1 μM. Calpain degrades titin, TnI, and TnT and has been shown to release α-actinin from the Z-line (Kasuga and Umazume, 1990; Geesink and Koohmaraie, 1999). Other work has shown that infusing $CaCl_2$ into pre-rigor lamb carcasses to raise the Ca^{2+} levels accelerated the on-set of postmortem tenderness (Koohmaraie *et al.*, 1988). Since 1989, a further 12 additional calpain homologues have been identified, the most documented of these is the skeletal muscle specific calpain 3 (Bartoli and Richard, 2005). Although it is considered to have very little proteolytic influence (Geesink *et al.*, 2005), Taveau *et al.* (2003) have demonstrated that titin is a substrate of calpain 3, or p94 as it is also known.

Calpains are thought to be the primary proteolytic system involved in meat tenderness for four important reasons (Jiang 1998); first, the ultrastructural degradation of postmortem muscle is very similar to that treated with calpain; second, the electrophoretic pattern of postmortem muscle is very similar to that of calpain treated myofibrils; third, the calpains are located in the Z-disk which is extremely susceptible to calpain-catalysed hydrolysis and fourth, the higher the level of calpain, the faster the rate of postmortem tenderisation. These reasons are supported by a study that showed that the injection of calpain inhibitors prevented tenderisation, whereas injection of cathepsin inhibitors did not (Uytterhaegen *et al.*, 1994).

Cathepsins

Cathepsins are lysosomal proteases that are not calcium dependent and remain within the organelle until cell death. There are a number of iso-enzymes, including cathepsins B, D, H, and L which proteolyse similar structures to calpain; cathepsins D and B break down myosin and other lower molecular weight proteins at postmortem pH (~<5.8), while cathepsin L primarily degrades troponins T and I (Jiang 1998). Cathepsin H has not been shown to have much effect on myofibrillar proteins (Ouali *et al.*, 1984).

There is still much debate surrounding the involvement of cathepsins in postmortem tenderisation. Although the enzymes are lysosomal, it is not clear whether or not they remain within this structure postmortem, or whether the decreasing pH weakens the walls of the organelles releasing cathepsins such as B, H, an L which have a pH optima of ~5.5-6.5 (Jiang 1998).

1.5 Cooking

1.5.1 The Effect of Cooking on Meat

One of the primary reasons why psoas major single muscle fibres were used in this study is their low collagen concentration. Although single fibres are enveloped in an endomysium, the psoas major has the least heat stable cross-links (Light and Bailey, 1983) and is the slowest to mature (King 1987). Single muscle fibres are also separated from the perimysium component, the connective tissue sheath that surrounds muscle fibre bundles and comprises the majority of the connective tissue in whole muscle samples. The contribution from this primary component of intra-muscular connective tissue is thought to influence both the texture and toughness of a cooked muscle sample. Texture is defined by the proportion of heat-stable cross-

links and toughness results from the compression of muscle fibres as collagen thermally denatures (Light and Bailey, 1983). It is well established that raw meat has a lower shear force than cooked meat (Dransfield and Rhodes, 1976). While the temperature increases and the meat begins to cook, there are two fundamental points where shear force increases and meat toughens. The first is thought to be between 40 and 60°C and the second between 65 and 80°C.

Depending on pH, myosin has been shown to denature between 57 and 65°C (Mutungi *et al.*, 1996). Davey and Gilbert (1974) suggested that the first increase in toughness is due to the denaturation of the myofibrillar proteins and that the second is a function of connective tissue shrinkage. However, Mutungi *et al.* (1996) showed that when a single fibre was heated, the forces required to fracture it did not change between 20 and 50°C, increased slightly between 50 and 65°C, and more than doubled when the temperature was raised above 65°C. Single muscle fibres lack perimysium, and so it is unlikely that increasing toughness at temperatures above 50°C is due to changes in connective tissue. Moreover, the increase in single fibre toughness (force needed to fracture) did not occur in two distinct phases as observed in whole meat. Mutungi *et al.* (1996) attributed the first observed increase in the toughness of meat to perimysium shrinkage, hence the reason why it failed to appear with respect to single fibre toughness. The second rise in toughness observed in whole meat was therefore a function of the denaturation of myofibrillar proteins. To support this theory, Christensen *et al.* (2000) demonstrated that the fracture strength of perimysial connective tissue increased from ambient to 50°C before decreasing between 50 and 80°C. In addition to this, differential scanning calorimetry (DSC) studies of rabbit skeletal muscle myofibrillar proteins, actin and

myosin, showed that myosin denatures between 50 - 60°C, while actin denatures around 80°C (Wright *et al.*, 1977).

1.5.2 Actomyosin Gel Formation

Upon heating, myofibrillar proteins undergo some degree of thermal denaturation. The most important proteins involved in this process are actin and myosin, which irreversibly denature to form a rigid three-dimensional gel. This transition is thought to begin at 30°C and reach a maximum at 60°C (Acton *et al.*, 1981). The rigidity of this gel depends on the environmental conditions in which the muscle proteins denature i.e. pH, ionic strength etc. (O'Neill *et al.*, 1993). Optimum cooking parameters are thought to be a pH of 6 and an ionic strength of 0.5 - 0.7 M.

Gelation is defined as protein aggregation involving the formation of continuous protein structures. As a result, the bulk water in such a structure is less mobile than free water, giving the gel its characteristic appearance and mechanical properties (Acton *et al.*, 1981). Gelation is a two-step process where aggregation follows denaturation resulting in an organised, continuous, three-dimensional structure (Wang *et al.*, 1990). In differing environmental conditions, some authors have referred to a type of random aggregation as being simultaneous protein coagulation and denaturation, and in some instances denaturation can follow coagulation (Acton *et al.*, 1981). The particular chain of events can be a function of the gelation parameters mentioned earlier as well as the heating rate, final temperature of the gel and sarcomere length (Mutungi *et al.*, 1995). These differences create the changes in gel texture and appearance. Wang *et al.* (1990) described this as a result of the change in ratio of viscous and elastic properties of the gel in response to the processing environment.

This theory was established by Yasui *et al.* (1982) when it was discovered that a considerable improvement in the rigidity of thermally-induced myosin gelation followed the addition of filamentous (F) actin to a mixture of myosin sub-unit fragments. The myosin sub-fragments had been prepared using chymotryptic digestion resulting in the formation of heavy meromyosin (HMM) and light meromyosin (LMM; Figure 1.3). It was concluded that a small amount of F-actomyosin formation had an enormous effect on the gel rigidity due to its ability to behave as a cross-link between the myosin sub-fragments. However, the addition of F-actin to solutions containing either HMM or LMM, but not both, had no observable effect. It appeared that a whole myosin molecule was needed in order for F-actin to have a significant effect. HMM contains the active actin binding site, but has no 'tail', while LMM is the 'tail', but in turn has no ability to bind actin. When present together, HMM binds to actin and the LMM provides a framework that can trap water and form a gel.

1.5.3 Effect of Sarcomere Length on Tenderness

During the process of cooking, sarcomere length greatly affects the stiffness of the fibre. The shorter the sarcomere length, the greater the maximum stress the fibre can withstand prior to yielding (Willems and Purslow, 1996). This is due to the number of cross-bridges formed prior to, or in the early stages of, cooking. The gel formed from the thermal denaturation of actomyosin is directly dependent on the number of these actin-myosin complexes, and the resulting stiffness is dependent upon the amount of gel formed. Mutungi *et al.* (1995) discussed the possibility that the '...fusing of the actin and myosin...to form a gel mass may provide a strong structure in the overlap regions accounting for increased force required to fracture the fibres'.

1.6 Aim and Outline

Although I am not claiming that single fibre stiffness is directly comparable to whole meat tenderness, a technique such as the one used in this thesis can isolate and evaluate components of muscle so our understanding of factors affecting tenderness and meat quality can be better understood. For example, sarcomere length, titin degradation and cooking are three factors that have a fundamental impact on the tenderness and eating quality of meat. In this thesis, a single fibre technique is used to evaluate the effect of these factors on the stiffness of single muscle fibres. If we can better understand how a single cell responds to changes in sarcomere length, protein degradation, and cooking, then we will begin to understand how these factors affect whole meat systems, and ultimately the quality of meat in the market place.

Using isolated single muscle cells provides an opportunity to study the response of myofibrillar structures without the confounding influence of extracellular components and interactions. The single muscle cells in this study were prevented from aging naturally by the addition of proteolysis inhibitors and the state of the fibres was controlled by physiological solutions that imitated both rigor and relaxed sarcoplasmic environments.

The aim of this thesis was to characterise the mechanical properties of ovine single skeletal muscle fibres in both the uncooked and cooked states. These properties are primarily dependent on the structural integrity of the protein lattice comprising muscle cells. The integrity and structure of these proteins change during thermal denaturation and post-mortem proteolysis. Selectively removing proteins and re-evaluating fibre stiffness should give a clear indication of the contribution of these proteins to the muscle cell's structural integrity during aging. Sarcomere length,

cooking and proteolysis are three variables associated with a change in the mechanical properties of the muscle. In this study all three variables were used as treatments applied to single skeletal muscle cells, with trypsin being used to simulate the effect of proteolysis. I hypothesise that each of these factors will have a measurable effect on the stiffness of single muscle fibres.

Chapter 2

The Contribution Of Sarcomere Length To The Stiffness Of Ovine Single Skeletal Muscle Fibres Before And After Cooking

2.1 Introduction

Various techniques have been developed to measure the attributes of cooked meat. The force required to shear a cooked sample of specific size perpendicular to the fibre direction is the most routine (Bratzler and Smith, 1963; MacFarlane and Marer, 1966), but resistance to strain or compression have also been explored (Lepetit 1991; Spadaro *et al.*, 2002). Shear force measurements are sensitive to the integrity of the myofibrillar structures within meat and correlate significantly with consumer perception of tenderness (Segars *et al.*, 1975, Toscas *et al.*, 1999).

Shear forces measured at rigor, or soon after (initial toughness), increase with the extent of contracture below rest length (Marsh and Leet, 1966; Davey and Gilbert, 1974). Sarcomere length is greatly influenced by factors such as the postmortem carcass chilling regime, which contributes to temperature-induced muscle contractures, and by carcass suspension, where the weight of the carcass can stretch some muscles (Willems and Purslow, 1996). Maintaining a muscle in a stretched state during the pre-rigor period produces lengthened sarcomeres, reducing initial shear force (Bouton *et al.*, 1973; Locker and Leet, 1975).

Since toughness associated with short sarcomeres persists even after clear evidence of proteolysis, the mechanism of toughening by short sarcomeres is probably

attributable to the increased overlap of thick and thin filaments (Wheeler and Koohmaraie, 1999).

The effect of sarcomere length on toughening is directly related to the varying degree of overlap of actin and myosin filaments after rigor mortis (Marsh and Carse, 1974). Bouton *et al.* (1981) demonstrated that cold-shortened meat with sarcomere lengths between 1.2 and 1.5 μm showed greatly increased peak shear force values when compared to muscles stretched pre-rigor to produce sarcomere lengths of between 2.7 to 2.9 μm.

The most likely explanation is that actomyosin coalesces to form more rigid structures of high tensile strength when the sarcomeres are short compared to when sarcomeres are long. Three lines of evidence suggest that the length of the I-band in cooked myofibrils defines the strength of the fibre. First, the length of the I-band is the main structural change in the sarcomere as the sarcomere length changes. Second, histological observations of meat stretched to break point typically demonstrate breaks in the I-band (Locker and Wild, 1982). Last, the toughening associated with short sarcomeres (1.6μm) is evident only after cooking (Dransfield and Rhodes, 1976). In the uncooked state, meat becomes increasingly more tender as it shortens, demonstrating the importance to tenderness of heat-induced denaturation of the actomyosin.

Stretching meat reduces overlap between thick and thin filaments, and the resultant large I band means that, at temperatures of 50°C or greater, the actin, and/or titin, would be responsible for linking the denaturing actomyosin filaments. Bouton *et al.* (1981) suggested that the initial yield point force values primarily represent myofibrillar strength, and that peak shear force includes the connective tissue

component. If this is indeed the case, they go on to discuss that the initial yield force values for raw and cooked meat were very similar below 55°C. At higher temperatures the initial yield point of the cold shortened samples became considerably greater demonstrating the decrease in tenderness of cold shortened meat during cooking.

Mechanical measurements of whole meat tissue defines the sum of all structural elements, but in particular the connective tissue and myofibrillar structures. Furthermore, these are derived from a heterogeneous mix of fibre types. In order to study the different structural components of muscle and meat in isolation, an alternative approach is to measure the structural properties of isolated individual muscle fibres. Mutungi *et al.* (1996) demonstrated that the behaviour of whole meat was reflected in the mechanical properties of individual fibres isolated after cooking; the stress at the fibres yield point decreased after proteolytic degradation, while fibres with contracted sarcomeres demonstrated increased yield stress (Mutungi *et al.*, 1995; Willems and Purslow, 1996; Christensen *et al.*, 2000).

A variation of the technique used by Mutungi *et al.* (1995) has been used in this thesis to describe the mechanical properties of isolated single muscle fibres and is based on measuring fibre stiffness as opposed to the fibres yield point. Fibre stiffness is measured by imposing small amplitude extensions to one end of an isolated muscle fibre and measuring the resultant stress by means of a force transducer connected to the other end of the fibre. Stiffness increases markedly when relaxed fibres are activated to produce cross-bridge cycling or when ATP is depleted and rigor bonds form. Stiffness is proportional to the number of attached cross-bridges (Yamamoto and Herzig, 1978; Higuchi *et al.*, 1995; Linari *et al.*, 1998). The principle source of compliance in a muscle fibre at rigor is therefore the

cross-bridges, although it has been argued that the thin filament also makes a significant contribution (Higuchi *et al.*, 1995; Linari *et al.*, 1998).

This chapter discusses the use of stiffness measurements, both before and following heating to 80°C, as a potential methodology for determining the structural changes associated with the tenderisation process of meat. For this purpose, we have used the recognised effects of sarcomere length on meat toughness as a comparative guide (Locker and Hagyard, 1963; Marsh and Carse, 1974).

2.2 Materials And Methods

All chemicals were obtained from Sigma unless otherwise stated.

2.2.1 Fibre Preparation

Lambs of standard Romney/Coopworth cross, aged between 6 - 12 months, were euthanised by an intravenous injection of excess pentobarbital (n = 7). This procedure minimises muscular activity and ensures a slow postmortem pH decline. The *psoas major* muscle was removed and bundles of approximately 1 mm x 3 mm wide and 10 mm in length were removed and tied to glass rods *in situ* to prevent shortening prior to excision (Eastwood *et al.*, 1979).

A chemical 'demembranation' solution (CDS) was prepared containing 40.5 mM KCl, 7.3 mM $MgCl_2$, 10 mM potassium ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (K-EGTA), 10 mM 3-[N-Morpholino]butanesulfonic acid (MOPS), 10 mM dithiothreitol (DTT), 5.4 mM Na_2ATP, 10 mM creatine phosphate (CrP), 15 units/ml of creatine phosphokinase (CPK), 2 mM NaN_3, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 g/L trypsin inhibitor (from chicken egg white) and 8 mg/L leupeptin (pH 7.1 at room temperature). The

chemical demembranation procedure was adapted from Bershitsky and Tsaturyan (1995), and included 90 minutes in CDS containing 0.5% triton X-100 (v/v), a 5 minute rinse in CDS, and 30 minutes in 25% glycerol-CDS. The procedures were carried out at room temperature (~20°C) and demembranated fibres were stored at -20°C in CDS containing 50% glycerol.

For the preparation of single fibres, a Petri dish with a rubber sealant coated interior was filled with CDS made up with 25% glycerol. A small bundle of fibres (~20-40 fibres) was removed from the glass rod, and pinned to the base of the petri dish. Single fibres were gently teased from these fibre bundles with sharpened forceps, clamped at each end with aluminium T-clips (Figure 2.1), and mounted between hooks for transfer to a fixing chamber as described by Linari *et al.* (1998).

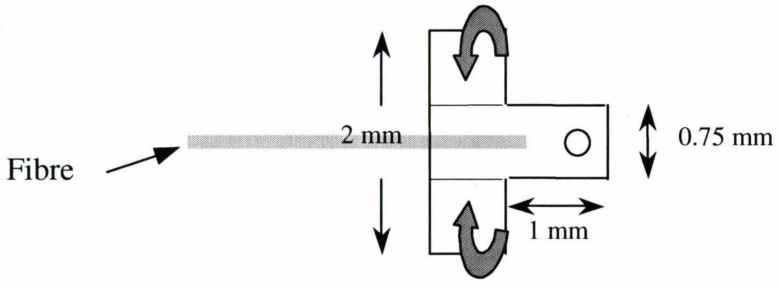

Figure 2.1 T-clip for mounting single fibres

The mounted fibre was immersed in a glass-sided tank of rigor solution (RigS; 10 mM MOPS, 98 mM KCl, 1.4 mM $MgCl_2$, 10 mM K-EGTA, 2 mM NaN_3, 0.1 mM PMSF, 0.1 mg/ml trypsin inhibitor, pH 7.0) and both fibre-clamp contact points were chemically hardened to minimise damage, compliance, and slippage at the attachment point of fibre and T-clip. This was done by applying a thin stream of the following aqueous solution to each end of the fibre; 25% glycerol, 4% glutaraldehyde, ~0.5% (w/v) Coomassie Blue R-250 – the Coomassie Blue R-250 stained the fibre and allowed identification of the chemically hardened, or 'fixed',

portion. The fibre was placed back in the dissecting solution, the T-clips cut off, and new clips attached to the fixed ends, leaving a small portion of the fixed end still exposed to produce unfixed fibre lengths of approximately 2.5–3.5 mm (Figure 2.2).

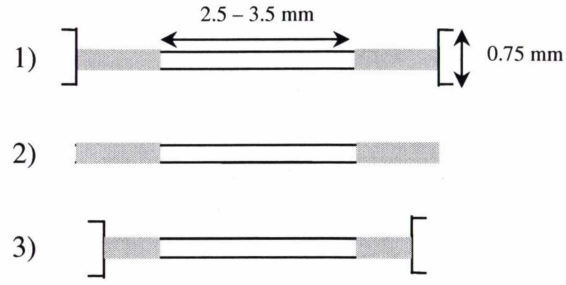

Figure 2.2 Fibre end treatment. Dark region = fixed.

1) After glutaraldehyde treatment of fibre ends

2) Cut off T-clips

3) Apply new T-clips

2.2.2 Experimental Set-up

The prepared fibre was mounted on a modified revolving stage of an optical microscope which contained five temperature-controlled troughs maintained at 7°C. The fibre was initially placed into relaxing solution (RelS; 10 mM MOPS, 90 mM KCl, 2.7 mM $MgCl_2$, 10 mM K-EGTA, 1.7 mM Na_2ATP, 2 mM NaN_3, 0.1 mM PMSF, 0.1 g/L trypsin inhibitor and 8 mg/L leupeptin, pH 7.0). The T-clip of one fibre end was mounted on a hook attached to the lever arm of a moving magnet rotary motor (308B, Cambridge Technology Inc.), while the other end was connected to a force transducer (AE801, Sensonor, Norway; resonant frequency 12 kHz). The T-clips were mounted on hooks made from short lengths of electrolytically sharpened tungsten wire (diameter 50 μm); one was glued to the

motor's lever arm and the other to the force transducer. The oscillating lever arm was driven by an arbitrary waveform generator and the output signal digitised at 2000 samples/s (Labview™, National Instruments). The output RMS was calculated using graphical data analysis software (DADiSP™, DSP Corporation).

2.2.3 Sarcomere length measurement

Fibre sarcomere length was adjusted to the desired length (+/- 0.05 μm) in RelS using a micromanipulator on the lever arm motor. Fibres were then placed in RigS for 2 minutes and allowed to stiffen. The distance between the mounting hooks was adjusted so the fibre was neither slack nor under tension. The fibre diameter and length were then measured. If sarcomere lengths below resting length were required, fibres were shortened by immersion in a 2,3-butanedione monoxime (BDM) activating solution (RelS, 20 mM calcium-EGTA and 50 mM BDM). In the presence of ATP, calcium produced uneven contractions and fibre damage, even at low concentrations. The inclusion of BDM decreases force generation by partially inhibiting crossbridge force production and the weak contractions minimise the risk of fibre damage (Higuchi, *et al.*, 1995). Fibres were first submerged in BDM activating solution, and the length of the fibres manipulated until the desired sarcomere length was reached. The sarcomere length was set by removing the fibres from the BDM activating solution and placing them into a Ca-Rigor-BDM solution for two minutes (RigS, 20 mM calcium-EGTA and 50 mM BDM). Fibres were then submerged in RigS for a further two minutes before measurements were made. The sarcomere length and diameter of the fibres were determined both before and after cooking using an eyepiece graticule at 600x magnification. The fibre was assumed to be essentially cylindrical (Zhao and Kawai, 1994; Willems and Purslow, 1996; Halsey 1999) and the average measurement of the diameter of the fibre at three sites

along its length was used to calculate the cross-sectional area. The length of the fibre was determined using a stereo microscope and graticule at 30x magnification.

2.2.4 Fibre Typing

To reduce the natural physiological variability between fibres, only fast-type fibres were used in this study. Fibres were selected from their response to strontium, to which slow fibres have a much higher sensitivity (Moisescu and Thieleczek, 1978; Kambara 1994). Fibre typing in this study was carried out according to West *et al.* (1999): a range of pSr^{2+} solutions was made by combining varying proportions of EGTA and Sr^{2+} solutions (EGTA solution; 60 mM N-[2-hydroxyethyl]piperazine-N'-[4-butanesulfonic acid] (HEPES), 10 mM magnesium oxide (MgO), 50 mM EGTA, 1.6 mM KOH, 8 mM ATP, 10 mM CP, 15 units/ml CPK, 1 mM NaN_3, 0.1 mM PMSF, 0.1 g/L trypsin inhibitor, and 8 mg/L leupeptin, pH 7.1. Sr^{2+} solution; 60 mM HEPES, 8.5 mM MgO, 50 mM EGTA, 40 mM strontium carbonate ($SrCO_3$), 1.6 mM KOH, 8 mM ATP, 10 mM CP, 15 units/ml CPK, 1 mM NaN_3, 0.1 mM PMSF, 0.1 g/L trypsin inhibitor and 8 mg/L leupeptin, pH 7.1). After exposing a number of both fast and slow fibres to a range of pSr solutions (Figure 2.4), a pSr^{2+} of 5.98 was selected as a solution which induced a 20% contraction in slow-type fibres and no response at all from fast-type fibres. Fibre type identification was validated by Western blot using a monoclonal antibody to skeletal myosin slow-type (clone no.NOQ7.5.4D; Figure 2.5).

2.2.5 SDS-PAGE and Western Blotting

In order to perform western blots, proteins were denatured and separated using a continuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) buffer system (Weber and Osborne, 1969).

2.2.5.1 SDS-PAGE

The single muscle fibres were removed from the microscope stage and placed in a microtube with 7.5 µl of sample buffer (25 mM Tris-HCl, 75 mM glycine, 2 mM ethylenediaminetetraacetic acid [EDTA], 2 mM dithiothreitol [DTT], 2% sodium dodecyl sulphate [SDS], pH 8.8). This preparation was then stored for up to 6 weeks at -20°C before being denatured at 50°C for 20 min. This temperature was lower than that typically used for SDS-PAGE, however, a trial using purified myofibrils demonstrated substantial titin degradation following electrophoretic preparation at higher temperatures (see Figure 2.3). After the denatured sample had cooled, 2.5 µl of sucrose buffer (25% sucrose, 0.0025% bromophenol blue) was added to the preparation and all 10 µl was loaded onto a 4% electrophoresis gel. The gel was prepared ahead of time by gently, but thoroughly, mixing 3 ml of milli-Q H_2O, 1.25 ml 1.5 M Tris HCl (pH 8.8), 670 µl acylamide:N,N'-bis-methylene acrylamide = 100:1 (wt/wt), and 50 µl 10% SDS. Immediately prior to gel casting, 75 µl of 10% ammonium persulfate (APS) and 15 µl N,N,N',N'-tetramethylethylenediamine (TEMED) were added, mixed gently for a few seconds, and then pipetted between clean glass plates contained in the casting frame. The mixture was allowed to set for a minimum of 1 h at room temperature or at 4°C overnight.

Once set, the gel was removed from the casting frame, loaded into a Mini Protean III Cell System (Bio-Rad Laboratories Pty Ltd), and one sample loaded per well (once denatured and cooled). The gel was run at a constant current of 7 mA until the dye front had electrophoresed from the base of the gel.

Gels were removed from the cell system, and either silver stained for visualization of the protein bands (Silver Stain Plus, Bio-Rad Laboratories Pty Ltd), or proteins were transferred to polyvinylidene diflouride (PVDF) membranes.

2.2.5.2 Western Blotting

Transfer of electrophoresed proteins involved electroeluting proteins onto a PVDF membrane using a semi-dry method.

A piece of PVDF membrane was cut to the size of the gel and marked in the lower right-hand corner for later orientation. The membrane was wetted briefly in methanol, and then soaked in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol made up to 500 ml, pH 8.3) for 2 to 5 min. Six pieces of filter paper were cut to the size of the gel, making sure that the filter paper and membrane were not larger than the gel. The filter paper was pre-soaked in transfer buffer. The surface of the Trans-Blot Semi Dry Blotting Unit (Bio-Rad Laboratories Pty Ltd) was wetted with milli-Q H_2O and three of the soaked filter paper pads were placed onto the unit. The PVDF membrane was placed on to the filter paper pads and a pipette tip, or similar, was used to roll out any air bubbles. The gel was placed onto the membrane and the other three filter paper pads on top of the gel, rolling the stack again to remove any air bubbles. Western blots were run at 35 mA for approximately 1.5 h. After electroelution was complete, the PVDF membrane was removed and washed in Tris-buffered saline (TBS; 5O mM Tris pH 7.3, 150 mM NaCl, made up to 500 ml in milli-Q H_2O). The membrane was blocked for 1 h at room temperature using 50 ml of 5% (wt/vol) powdered non-fat milk in TBS (pH 7.3). Diluted (1:2000 in 10 ml TBS) myosin slow primary anti-body (clone no.NOQ7.5.4D) was added and incubated on the membrane as required (usually 30-60 min at room temperature). The membrane was quickly washed twice in TBS-T (TBS, 0.05% Tween-20), then three more times for 5 min each. Diluted secondary antibody (1:3000 in 10 ml TBST) was incubated on the membrane for 30-60 min at

room temperature. The membrane was again rinsed five times in TBST for 5 min each.

Using ECL Plus western blot detection system (Amersham Pharmacia Biotech), 1 ml of Solution A was mixed with 25 µl Solution B as per the manufacturer's instructions, pipetted on to the membrane and incubated for 5 min. Excess was poured off and the membrane and placed onto a cut-open plastic bag, closed and taped into an exposure cassette. Membranes were developed and fixed in a darkroom as per the manufacturer's instructions.

2.2.6 Stiffness Measurement

The stiffness of fibres in rigor was measured using small amplitude stretches in the form of sine wave oscillations applied by the motor arm (Higuchi *et al.*, 1995; Linari *et al.*, 1998). The amplitude of the applied oscillations was adjusted for the length of each fibre to produce an extension of 5 nm/half sarcomere (nm/h.s.). An initial tension of 20 kPa was applied to the fibres to remove any filament slack before imposing an oscillation of 5 Hz. This frequency was chosen by running a frequency sweep from 10 Hz to 500 Hz while the fibre was mounted; a resonant frequency developed at >300 Hz, while at >10 Hz, inertia of the lever arm reduced the maximal displacement (Figure 2.7). To establish which frequency gave the most consistent, maximal movement, both 5 and 10 Hz were compared (Figure 2.8). 5 Hz was eventually selected as the frequency which allowed the most consistent lever arm movement.

The response data from each oscillation were used to calculate the root mean square (RMS) value. Three values were averaged to give a mean RMS value for each fibre. The mean RMS value was converted into stiffness (N/m^2) using the expression given

below. The intercept and slope given in the expression were obtained from the force transducer calibration curve (Figure 2.9).

Stiffness is normally defined by Young's Modulus, $Y = (F * L_0)/(A_0 * \Delta L)$; where F is the force applied, L_0 is the initial length, A_0 is the original cross-sectional area, and ΔL is the change in length. I have chosen not to use Young's Modulus to define "stiffness" in this thesis for a number of reasons; first, muscle fibres are not a homogenous material and their response to strain is not linear; second, soft biological materials are highly extensible, non-isotropic and follow the principle of finite elasticity; third, the strain applied to the fibre is not length dependent, but dependent on the number of sarcomeres and is intended to be constant per half sarcomere (5 nm/h.s.). Therefore the term "stiffness", as used in this thesis, is defined by the expression given below, and is given in N/m^2;

$$Y\ (N/m^2) = \frac{\text{(mean RMS-intercept)/slope/fibre cross sectional area} * (9.81 * 10^{-6})}{\text{fibre length/sarcomere length} * 2}$$

To gain an understanding of the contribution of titin to the stiffness of relaxed fibres, a continuous stretch of 932 µm at a rate of 73 µm/s was imposed on uncooked fibres bathed in RelS. The fibre length and resting sarcomere length were recorded so the stress response could be correlated with the increasing length of the sarcomeres.

The fibres underwent the non-destructive oscillations before and after cooking, and prior to being removed they underwent two consecutive stretches. The primary yield point of fibres is defined as the point at which the slope of the increasing stress response decreases and irreversible damage has been done. Fibres cooked at 80°C were measured using a strain of 20% of initial resting sarcomere length. The first of these stretches was used to calculate the initial yield point as described above, while

the second stretch was used to demonstrate the presence of damage as evidenced by the hysteresis between the consecutive stretches. The stress and strain at yield were obtained from the inflection point calculated from the second derivative using graphical data analysis software (DADiSP™, DSP Corporation).

2.2.7 Heating procedure

To mimic the cooking process, a separate heating trough was constructed on a miniature heating element and the whole assembly mounted on the microscope stage. The temperature was controlled by a copper/constantan thermocouple (T type, 0.127 mm, Omega, USA) connected to a temperature controller. For all heating experiments, fibres were immersed in RigS, the temperature of which was increased from ambient (~20°C) to 80°C in 6.5 min. This temperature was maintained for a further 3.5 min. Changes in isometric fibre tension during heating were also recorded. For subsequent analysis, fibres were transferred to RigS at 7°C.

In this study, the cooking parameters were kept constant at the same simulated physiological conditions used in the uncooked state. This was done in order to determine the effect of the proteolytic treatment on actomyosin gel formation and filament rigidity after cooking, without introducing an environmental variable. A separate study using the single fibre technique could be used to define the mechanical differences in gel formation as a result of altering the environmental parameters.

2.2.8 Statistical Analysis

Each fibre was allocated a sarcomere length at random and measurements were made on that fibre in both the uncooked and cooked states. Although the stiffness of each fibre was measured in the raw and cooked states, sarcomere length was an

independent measure and only one sarcomere length was represented by any given fibre. This was done to reduce the potential damage to the fibre by repeatedly altering the sarcomere length. In addition to this, each fibre could only be heated once at any given sarcomere length so it was felt that for consistency of data, each fibre would only be used to represent one sarcomere length.

The data for each sarcomere length in each treatment were compared using a 2-way ANOVA General Linear Model and the source of any significant difference was determined by a post-hoc multiple pair-wise comparison procedure (Tukey).

2.3 Results

Figure 2.3 demonstrates the thermal instability of titin indicated by heating samples of a purified myofibril preparation under denaturing (in the presence of SDS) or non-denaturing conditions using two different denaturation temperatures and durations for SDS-PAGE. This was done to establish the best denaturation temperature for resolving heat-labile titin. The lack of any titin banding in any of the last 4 lanes suggests that titin was degraded during heating to 100°C for 3 min in denaturing sample buffer. The first four lanes have obvious titin bands that have been resolved by denaturing the samples at 50°C for 20 min following pre-heating at temperatures up to 75°C. The fifth lane indicated that pre-heating titin to 80°C before addition to sample buffer degraded the protein to the point where T_1 and T_2 could not be reasonably resolved. Based on these findings, all gels were denatured at 50°C for 20 min unless otherwise stated.

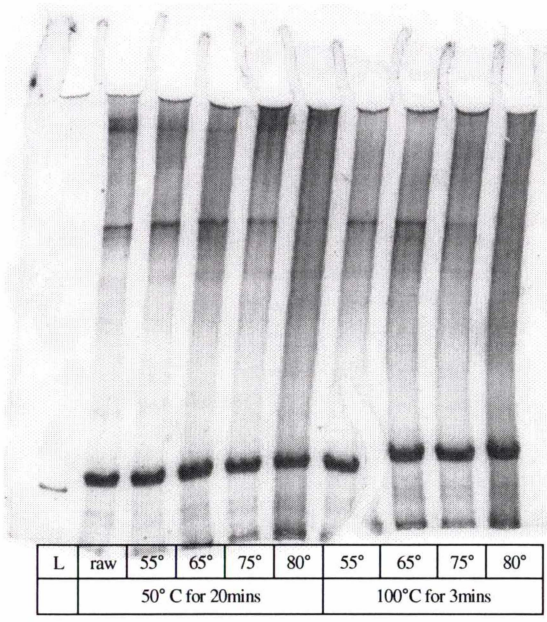

Figure 2.3 Comparison of denaturation temperatures prior to loading. Samples of a purified myofibril preparation were heated to 55, 65, 75, or 80°C for 10 min prior to being added to the sample buffer. Samples were then heated to either 50°C for 20 min or 100°C for 3 min to denature proteins in the presence of SDS. Raw indicates that the sample was not heat treated prior to denaturation.

Strontium activation was used to identify fibre type while fibres were mounted on the microscope stage. Figure 2.4 displays the mean activation curves for fast (blue; n = 10) and slow (pink; n = 8) ovine fibre types. A pSr^{2+} of 5.98 was chosen for fibre typing mounted fibres as this concentration would illicit a response of approximately 40% of full activation in slow fibre types while fast fibres would not respond to this concentration at all. To validate this method, fibres that had been identified using pSr activation, were loaded onto a gel and a western blot was done using myosin slow-type clone no.NOQ7.5.4D (Sigma). Western blotting confirmed the identifications made using strontium activation (Figure 2.5).

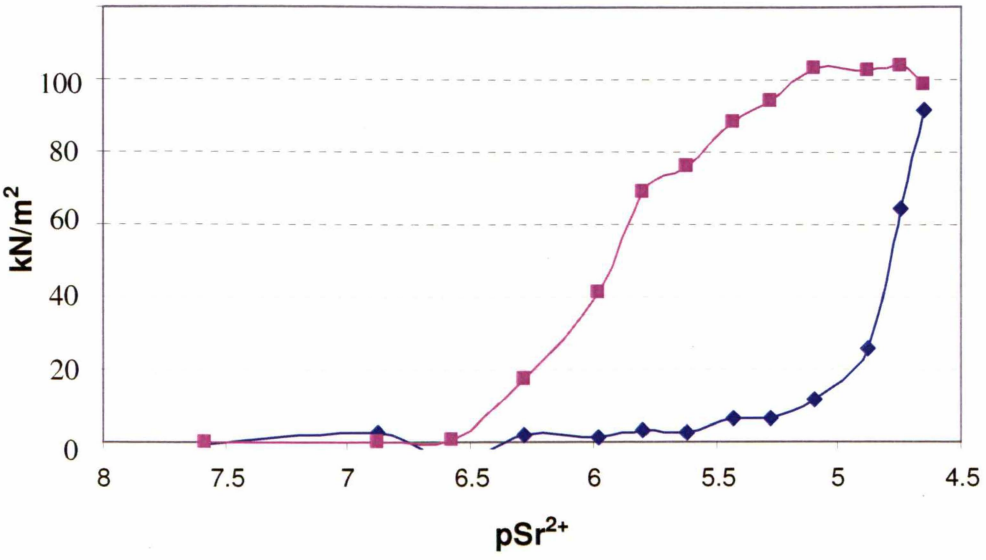

Figure 2.4 Mean strontium activation curve for slow- (pink; n=8) and fast- (blue, n=10) twitch fibre types.

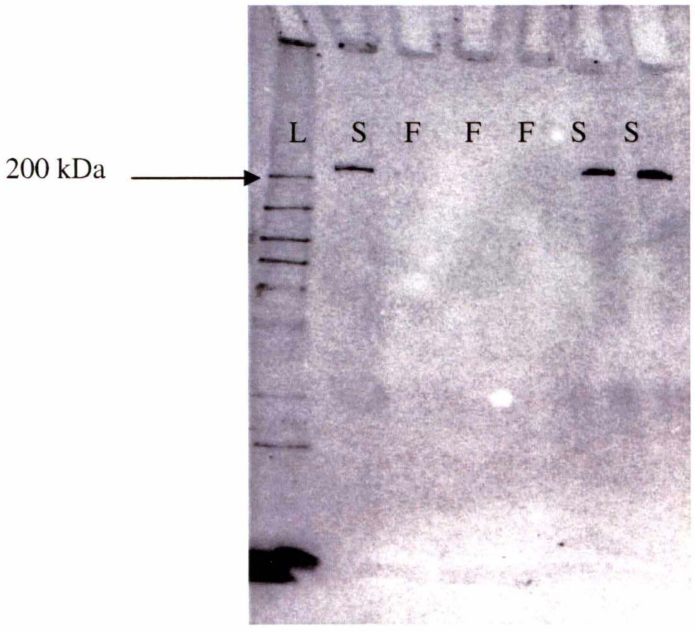

Figure 2.5 Validation by western blotting of in vitro non-destructive fibre typing based on Sr2+ activation patterns as indicated in Figure 2.4. Western blotting confirmed identification (using myosin slow clone NOQ7.5.4D).

L = ladder, S = slow and F = fast

This study evaluated a methodology for measuring the stiffness of uncooked and cooked isolated muscle fibres of defined glycolytic type (fast twitch), and assessed its potential as a means of assessing the relationship between sarcomere length and stiffness. To do this a number of measurement parameters had to be defined. Figure 2.6 demonstrates the response of the fibre and the deflection of the lever arm in response to a 10 to 500 Hz frequency sweep. Resonance appeared in the fibre response at a frequency of ~300 Hz. In addition, the deflection of the lever arm appeared to drop at ~75 Hz. This was due to the weight of the lever arm limiting extension. At 10 Hz a 0.05 V oscillation still resulted in inconsistent output amplitude of ~0.04 - 0.045 V (Figure 2.7). An oscillating frequency of 5 Hz was finally utilised as the consistency of the amplitude was not affected by the weight of the lever arm (Figure 2.8).

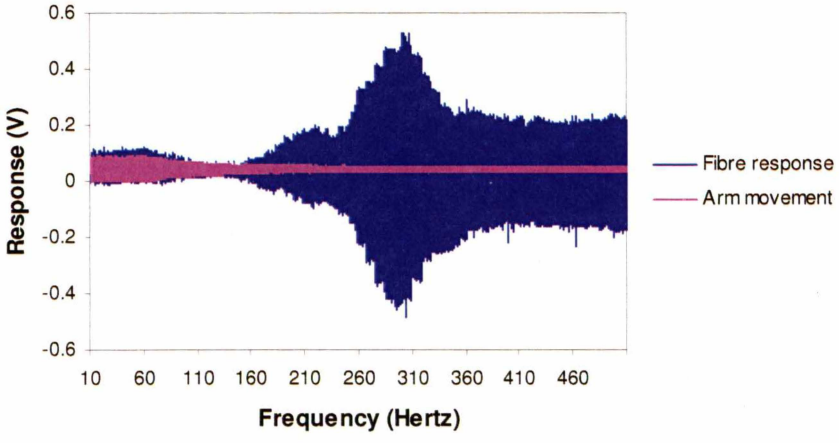

Figure 2.6 Frequency sweep to determine measurement parameters. Fibre response is measured in terms of force transducer output voltage.

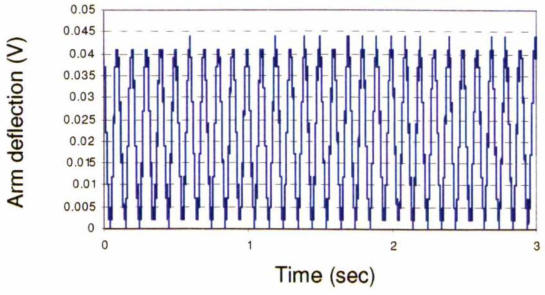

Figure 2.7 Response of the lever arm to a 10 Hz sine wave. Input voltage = 0.05 V.

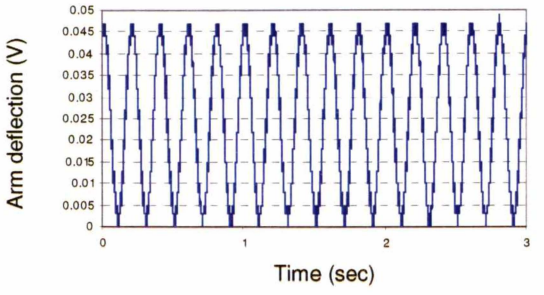

Figure 2.8 Response of the lever arm to a 5 Hz sine wave. Input voltage = 0.05 V.

The force transducer was calibrated (using weights) to allow calculation of the expression given in Section 2.2.6. Calibrations were updated regularly and applied to the subsequent calculations. Figure 2.9 shows an example of a calibration curve used.

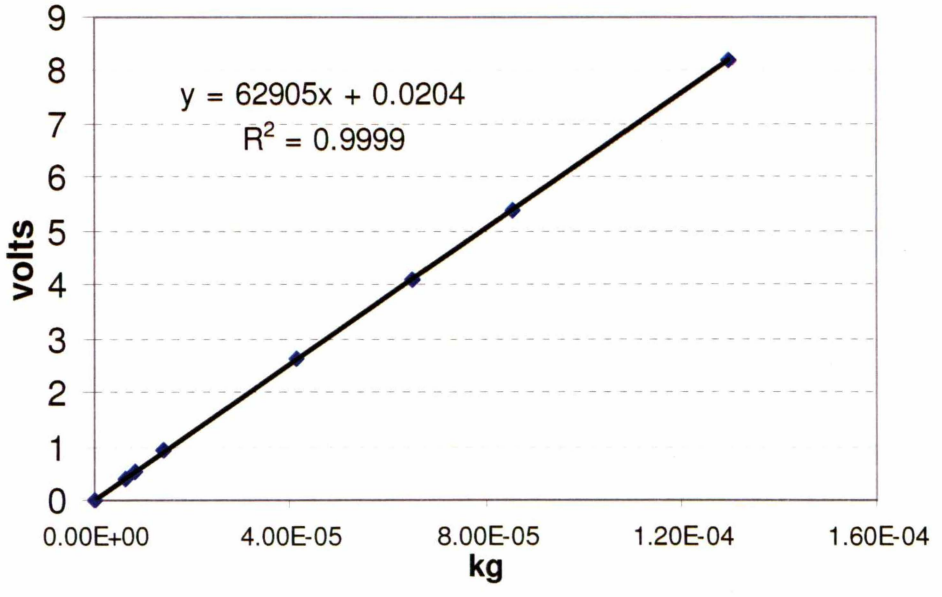

Figure 2.9 Example of a calibration curve used to enable calculation of stress.

2.3.1 Uncooked Fibres

To determine the contribution to fibre stiffness of non-contractile, myofibrillar components, the stress response of a single, uncooked fibre in the relaxed state (ATP present) was measured using continuous stretch of 932 μm, or 32% of the total resting length, at a rate of 73 μm/s. Until the continuous extension stretched the fibre to 2.5 μm, the tension produced by a relaxed fibre was below the detection level of the force transducer. A tension first became evident at approximately 2.5 μm and increased to reach an equipment limited maximum of 43.8 N/m^2 at a sarcomere length of 3.15 μm (Figure 2.10).

The muscle fibre response to small amplitude, sinusoidal strains, expressed as the average root mean square (RMS) of three responses, for all in-rigor fibres at each sarcomere length is given in Figure 2.11. The stiffness of fibres in the uncooked and cooked states was measured at sarcomere lengths of 1.6, 1.8, 2.1, 2.7, 3.0 and 4.2 μm. The stiffness of uncooked muscle fibres in response to different sarcomere lengths showed a bimodal pattern. At resting length (2.1 μm), skinned fibres produced a measured stiffness of 19.4 $N.m^{-2}$/half sarcomere ($N.m^{-2}$/h.s.). Fibres with longer sarcomeres displayed lower stiffness at 2.7 and 4.2 μm (13.3 and 13.1 $N.m^{-2}$/h.s respectively), but these differences were not significant. At 3.0 μm, fibre stiffness was 19.8 $N.m^{-2}$/h.s, equivalent to that of rest length. Fibres with sarcomere lengths shorter than rest length showed no significant difference in stiffness (20.3 and 16.4 $N.m^{-2}$/h.s at 1.8 and 1.6 μm respectively). When comparing 2.1 and 2.7 μm, a drop in fibre stiffness was evident as filament overlap decreased from 100% to 76%, but again this difference was not significant. Unexpectedly, the stiffness appeared greater in fibres with sarcomere lengths of 2.7 compared to 3.0 μm, when the approximate filament overlap was only 55%.

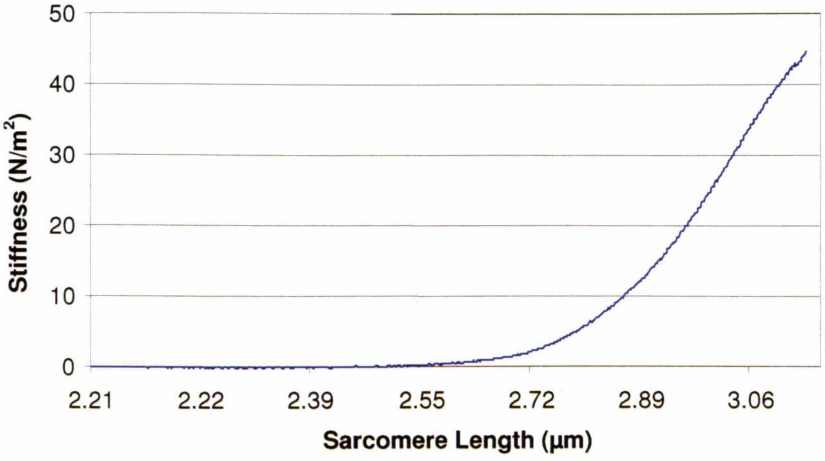

Figure 2.10 The response of a relaxed fibre stretched to a sarcomere length of 3.15 µm demonstrates the increasing contribution of titin to fibre stiffness at extended sarcomere lengths.

2.3.2 Cooked Fibres

2.3.2.1 Effect of sarcomere length on stiffness

In addition to measuring the characteristics of fibres in the uncooked state, the procedures reported here also allowed individual muscle fibres to be heated to 80°C and the mechanical characteristics of the cooked fibre to be measured. Heating fibres to 80°C significantly reduced the stiffness compared to the uncooked state ($P<0.05$; Figure 2.11). The various stiffness responses of fibres at different sarcomere lengths differed from that of uncooked fibres: relative to resting length (2.1 µm), increasing sarcomere lengths produced little change in stiffness, although a slight increase in stiffness was measured in fibres with a sarcomere length of 2.7 µm compared to 3.0 µm (5.0 $N.m^{-2}/h.s$ vs 8.9 $N.m^{-2}/h.s$ respectively). However, stiffness increased when sarcomere length was reduced below rest length: fibres with sarcomeres of 1.8 µm displayed an increased stiffness of 11.2 $m^{-2}/h.s$ compared to 7.0 $N.m^{-2}/h.s$ at rest length and a sarcomere length of 1.6 µm showed significantly increased stiffness (17.3 $N.m^{-2}/h.s$) when compared to 2.1 µm ($P = 0.05$).

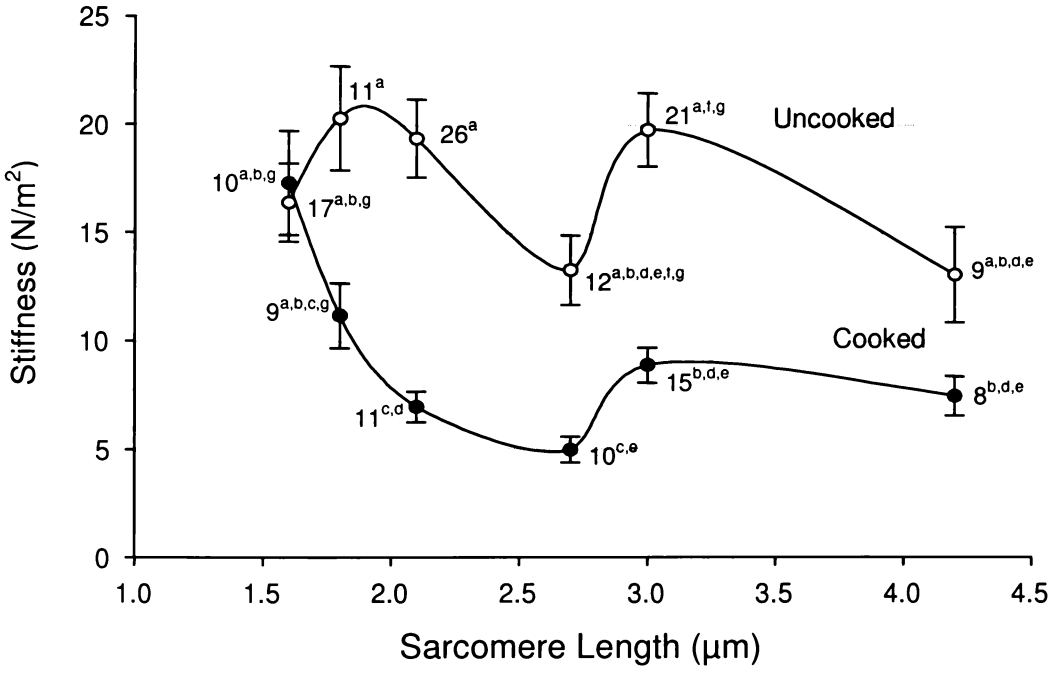

Figure 2.11 Stiffness of uncooked muscle fibres and fibres heated to 80°C then returned to 7°C before stress analysis. Data points are means ± SEM and n values are given next to their respective points. Different superscripts represent significant differences within and between uncooked and cooked treatments. Values with the same superscript are not significantly different.

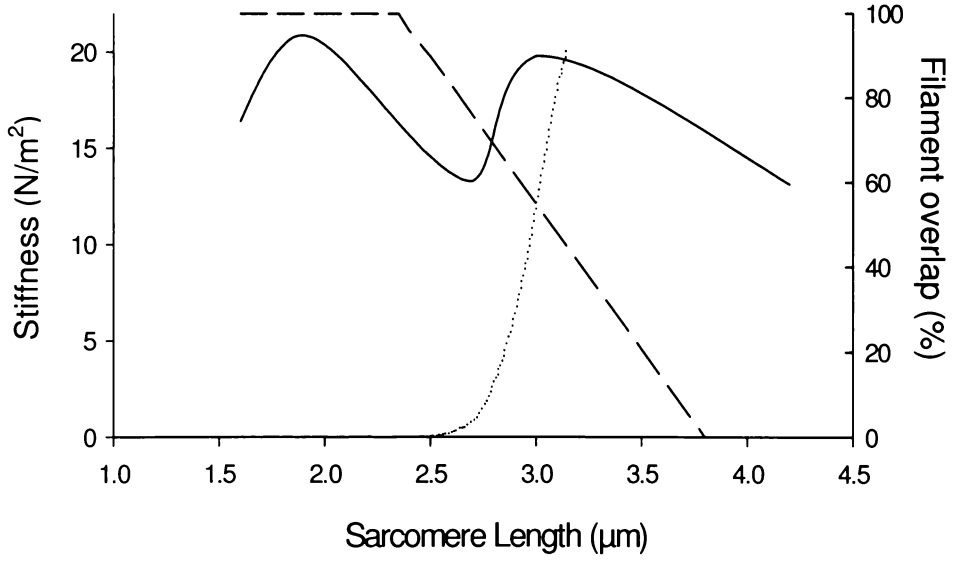

Figure 2.12 Stiffness of uncooked fibres (solid line) compared with calculated % filament overlap (dashed line), and the increasing stiffness of a relaxed fibre subjected to a continuous stretch (dotted line).

As in the uncooked fibre (Figure 2.12), the stiffness of the cooked single fibre increased between 2.7 and 3 μm, but the increase was not statistically significant. Figure 2.13 shows the difference in stiffness responses of fibres at various sarcomere lengths compared to the width of the I band (using an inverted abscissa) unlike the dashed line in Figure 2.12 which depicts the percentage of filament overlap.

2.3.2.2 Yield point characteristics of cooked fibres

In order to allow some comparison between the small oscillation stiffness of cooked fibres and the yield point during stretch, calculations were done on the yield point of cooked fibres that were stretched to 20% of their initial resting length. The first inflection or yield point of cooked fibres occurred in the fibres with a sarcomere length of 1.6 μm at an average strain of 2.3% (SEM = 0.102) of fibre resting length and a stress of 12.7 kN/m^2 (SEM = 0.998). This is not where the fibre breaks, but where the slope appreciably changes implying the onset of damage or a conformational change in the myofibres.

The yield point of fibres set at a sarcomere length of 2.7 μm occurred at 5.58% (SEM = 0.324) of fibre resting length and a stress of 41.6 kN/m^2 (SEM = 7.646). This yield point is at a greater strain implying that at longer sarcomere lengths the fibre was more elastic. This could be a function of the longer I-band. Both stress and strain yield points at the lengths discussed were statistically different from each other ($P < 0.001$).

2.3.2.3 Tension changes in fibres during heating

The procedure described here also allowed tension measurements to be made during heating to 80°C. At an average temperature of 62.1°C (SEM = 0.31, n = 23; Figure 2.14), the resting tension of the fibre underwent an abrupt increase. The peak

isometric force produced during heating reached an average value of 27.9 kN/m^2 for all sarcomere lengths below 3.8 μm (SEM = 1.8, n = 57). At sarcomere lengths of 3.8 μm or less, neither the temperature at which tension increased (P = 0.409) nor the peak force (P = 0.666) was affected by sarcomere length. At a sarcomere length of 4.2 μm, the thick and thin filaments do not overlap and a continuous tension is needed to maintain the sarcomere length during heating. This tension gradually decreased during heating and completely disappeared at a mean temperature of 65.7°C (SEM = 0.81, n=8, Figure 2.14). A typical example of the tension changes during heating of fibres held at a sarcomere length of 4.2 μm is shown in Figure 2.14. As sarcomere lengths were not statistically different from each other, the increase in fibre tension is represented by a single example (in this instance the sarcomere length is 2.1μm).

2.3.2.4 Change in Fibre Diameter

Fibre diameters were measured before and after heating to 80°C. The diameter of a heated fibre was on average 61.7% (SEM = 0.56, n = 95) that of an unheated one. Sarcomere length was not affected as fibres were constantly restrained.

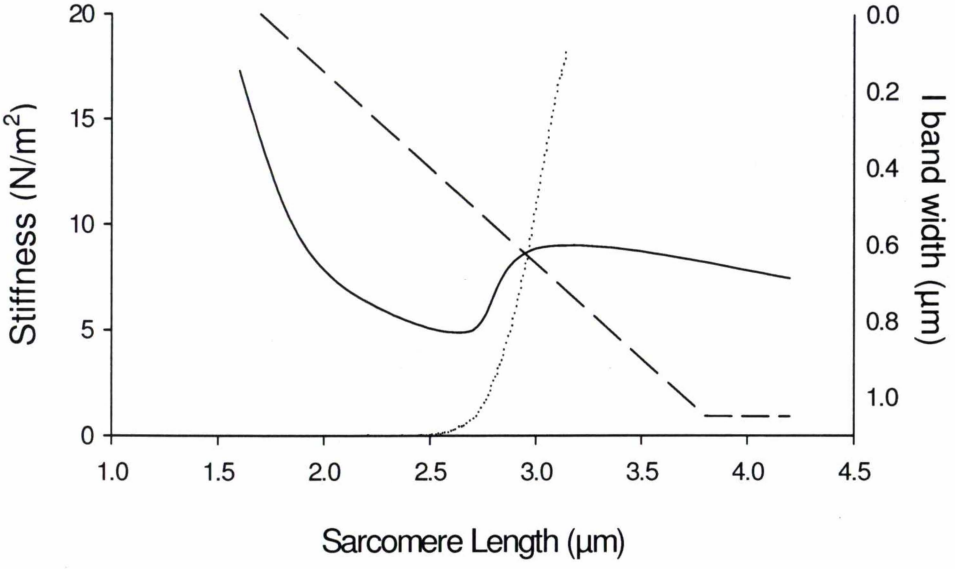

Figure 2.13 Stiffness of cooked fibres (solid line) compared with calculated I-band width (dashed line and inverted axis), and the increasing stiffness of a relaxed fibre subjected to a continuous stretch (dotted line)

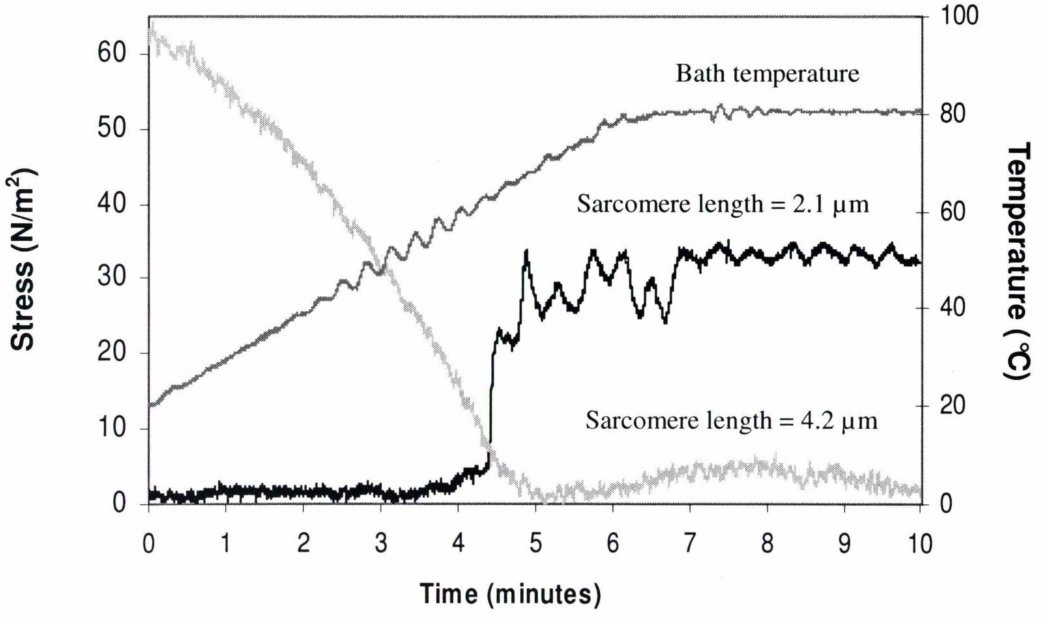

Figure 2.14 A representative example of tension onset (sarcomere lengths less than 3.0 µm), and tension reduction (sarcomere length = 4.2 µm) during heating to 80°C.

2.4 Discussion

2.4.1 Uncooked Fibres

Considering first the properties of uncooked fibres, their compliance, in response to small amplitude extensions, has previously been studied in actively contracting and rigor fibres (Yamamoto and Herzig, 1978; Higuchi *et al.*, 1995; Blangé *et al.*, 1997; Linari *et al.*, 1998). These studies have concluded that the stiffness of both active and rigor fibres is defined, primarily, by the number of bound cross-bridges. In rigor, for example, all cross-bridges that can attach are attached (Cooke and Franks, 1980). For this reason, the methodology used here adjusted the applied strain to the number of sarcomeres (or, by convention, half sarcomere, calculated from dividing the observed sarcomere length in to the measured fibre length) in order to keep the strain experienced by each attached crossbridge constant, rather than correct the strain for the length of the fibre. The use of Coomassie blue in the solution used to fix the ends of the fibres during the fibre preparation ensures that the functional length of the fibre can be accurately measured while also minimising the risk of artifactual compliance due to damage of the fibre at the point of attachment.

The proportion of bound cross-bridges can be calculated from sarcomere length. The length of the thick myosin-containing filament appears to be relatively constant across species, at 1.6 μm (Burkholder and Lieber, 2001), while the length of the actin-containing thin filament, though more variable than the thick filament, has been estimated at 1.1 μm from the centre of the z-line to the end of the thin filament in rabbit myofibres (Swartz *et al.*, 1993). Applying these values to ovine skeletal muscle, a sarcomere length of 2.35 μm represents 100% overlap (all myosin heads overlapping the thin filament), and I-band lengths are 0.325 μm. Sarcomere lengths

of 2.7 μm correspond to 76% overlap, whereas >3.8 μm extends the sarcomere beyond the point of any overlap. Sarcomere lengths of 2.1 μm and 1.8 μm both result in 100% overlap but I-band lengths differ at 0.2 μm and 0.05 μm respectively. Accordingly, stiffness responses in uncooked fibres should decrease proportionally as sarcomere length increases, but only fibres with sarcomere lengths greater than 2.35 μm, show decreasing stiffness as the percentage of actomyosin crossbridges begin to decreases. A sarcomere length of 2.1 μm was determined to be resting length for ovine muscle fibres. This was a result of measuring sarcomere lengths of fibres in RelS with no imposed tension. This determination is also supported by Marsh and Carse (1974) who measured bovine muscle resting length at 2.1μm.

The results presented in Figure 2.11 appear consistent with the calculations given above. The stiffness of the uncooked fibres with 100% overlapped cross-bridges at sarcomere lengths of 1.6 – 2.1 μm did not differ. It is evident therefore that this measurement procedure does not identify compliance of the I-band, since, within this range of sarcomere lengths, the number of attached cross-bridges remains constant but the I-band length varies. In this respect, stiffness or stiffness measurements are similar to shear force measurements of uncooked meat. The latter shows a linear decline in samples with proportionally decreasing sarcomere length, an effect that has been attributed to changes in fibre diameter: as sarcomere lengths become proportionally shorter, fibre diameter increases and the number of fibres in a unit area of meat decreases. Shear force consequently decreases, but calculating an appropriate correction for fibre volume results in shear force becoming independent of sarcomere length in uncooked meat (Dransfield and Rhodes, 1976). Since changes in fibre diameter in response to incrementally decreasing sarcomere lengths

do not affect stiffness measurements of individual fibres, an equivalent correction does not apply.

The bimodality of fibre stiffness in uncooked fibres reported here (refer Section 2.3.1) is similarly reported by Tawada and Kimura (1984). A possible explanation may relate to the contribution of titin: I confirmed in ovine m. *psoas major* fibres the observation previously made in other species that passive stretching of relaxed fibres results in the appearance of tension at sarcomere lengths beyond 2.5 μm (Figure 2.12) which increases markedly at further extension (Linke *et al.*, 1996). This event is attributed to stretching the titin molecule (Funatsu *et al.*, 1990; Wang *et al.*, 1991; Higuchi 1992; Kellermayer *et al.*, 1998). The coincidence of increased stiffness of rigor fibres with elastic extension of titin in both the uncooked and cooked states (Figure 2.12 and Figure 2.13) at a sarcomere length of 3.0 μm may therefore reflect an additional contribution of the titin molecule to the stiffness generated by the bound cross-bridges. The reduction in stiffness when sarcomere lengths are further extended to 4.2 μm might be attributed to the complete loss of filament overlap (which occurs at 3.8 μm), negating any additional increase in stiffness associated with further extension of titin.

2.4.2 Cooked Fibres

The tensile properties of whole cooked meat are generally recognised as the sum of contributions of connective tissues and the varying myofibrillar properties of multiple fibre types (Bendall 1975; Christensen *et al.*, 2000). The purpose of this technique was to evaluate the structural properties of individual muscle fibres under raw and cooked conditions using a non-destructive stiffness measurement.

The effects of short sarcomere length on toughness of whole meat are evident only after cooking (Purchas 1973; Davey and Gilbert, 1975; Rowe 1977) and, as in whole meat, cooked single fibres exhibited a pronounced increase in stiffness of fibres with sarcomere lengths of less than 2.1 μm. This increased stiffness of cooked single in proportion to the decrease in sarcomere length is a clear analogy to the increased toughness of whole meat at short sarcomeres (Marsh and Carse, 1974; Smulders *et al.*, 1990).

Marsh and Carse (1974) also demonstrated an increase in toughness of cooked whole meat at an average sarcomere length of just over 2.6 μm. This is comparable to the present results with single fibres, given the recognised difficulty of measuring sarcomere length accurately in whole meat. The appearance of increased stiffness of single fibres, and toughness in whole meat (Marsh and Carse, 1974) at the extension at which titin stiffness becomes apparent is again very suggestive of a role for this structural protein in defining the textural characteristics of cooked meat.

Comparing fibres with sarcomere lengths of 2.7 μm to those with 4.2 μm did not show a significant reduction in stiffness, which contrasts with the shear forces of whole meat (Marsh and Carse, 1974). Shortening the sarcomere length below 2.1 μm does not affect the number of cross-bridges but reduces the length of the I-band, indicating that a shorter I-band contributes to increased stiffness of single cooked fibres. However, I-bands longer than those in fibres at rest length did not show reduced stiffness, suggesting that the link between stiffness and I-band length is not linear, or possibly concealed by the contribution of titin.

A further objective of this research was to compare the stiffness measurements with the conventional shear force measurements of whole meats. Shear force analysis of

cooked meat typically measures the stress at fracture to provide an index of tenderness: this is clearly distinct from the stiffness measurement used here. In principle, there is no necessary association between stiffness as measured by small amplitude extensions and ultimate yield stress, although there is no reason why such a relationship should not exist. Spadaro *et al.* (2002) found that force at fracture and resistance to deformation (stiffness) are related structural properties of heat denatured (cooked) meat: they determined that meat stiffness, measured by way of energy dissipated and initial and final stiffness during the imposition of a 3% strain to whole meat were highly correlated with tenderness, as defined by sensory evaluation. In the experiments reported here, we used a well recognised model of cooked meat toughness, shortened sarcomeres, to demonstrate a potential link between toughness in whole meat and stiffness in single fibres.

The tension increase that became evident at around 62°C during heating corresponds to the denaturation temperature of myosin (Wright *et al.*, 1977). This denaturation is believed to be responsible for the myofibrillar component of cooking-induced toughness (Mutungi *et al.*, 1996; Christensen *et al.*, 2000), and is distinct from a separate, heat-induced toughening associated with denaturation of perimysial connective tissue (Davey and Gilbert, 1974).

Heating also produced a very distinctive yield pattern in the fibre's responses to stretching. Uncooked rigor fibres can typically be extended about ~2-5% of rest length before yielding (Mutungi *et al.*, 1995), whereas heated fibres could be stretched to greater than 5% of their initial length before yield point is reached. When a strain of 4% was applied, followed immediately by a return to rest length and the strain then re-applied, the stress-strain curves remained the same. When a strain of 8% was applied to the fibre, the stress-strain relationship changed when the

stretch was repeated. When fibres are subjected to paired stretches in stepwise increments between 4 and 20%, each increment of increased stretch from 8% caused the stress-strain relationship to change in the second of the paired stretches. This lack of reproducibility demonstrates that an irreversible structural modification of the fibre is taking place. The ultimate yield or break point, although not measured in this study, is therefore the end of a process that begins at applied strains of >4%.

The results reported in this Chapter are similar to those reported by Mutungi *et al.* (1995) except that these authors reported greater yield strains of 10-20% of fibre length for cooked fibres. This disparity could be due to differences in fibre preparation. These authors used fibres isolated after cooking, and the evidence of irreversible changes we report at strains of only 5%-8% of resting length (100-160 μm extension of a 2 mm fibre) suggests that the isolation of fibres after cooking may produce some damage to the fibres during preparation.

Figure 2.13 shows an increase in the stiffness of cooked fibres at the sarcomere length at which titin tension becomes evident. What is surprising is that the effects of titin should remain apparent after cooking: during the course of which, titin is expected to degrade. For example, Fritz *et al.* (1992) demonstrated that titin began to degrade and fragments became evident on sodium dodecyl polyacrylamide gels at a temperature of 59°C, with further gradual degradation occurring until 72°C when no intact titin could be detected. That titin undergoes major transformations during the heating process is demonstrated here by the isometric tension changes in fibres stretched to 4.2 μm (Figure 2.14). Since cross-bridges between thick and thin filaments cannot form at this sarcomere length, an initial tension, produced by the stretching of the titin molecule, must be applied on the fibre to maintain the sarcomere length. During heating, this tension gradually declines, starting at

approximately 20°C and disappearing at, typically, 65°C. This reflects the temperature range over which conformational changes and/or loss of structural integrity occurs to the titin molecule during heating. It is not clear at this stage how to reconcile the effect of titin on fibre stiffness with the evident degradation of the molecule during heating (Fritz *et al.*, 1982). This degradation may suggest a role of titin, or some of its degradation products, on the structural characteristics of the actomyosin gel that forms during heating.

The reduction in fibre diameter was not affected by sarcomere length. Bendall and Restall (1983) heated isolated muscle fibres to 90°C and also found a decrease in myofibre diameter. However, in their experiments, the fibre fragments failed to shorten during heating suggesting that very minimal tension development would have occurred. This lack of tension development is in clear contrast to the present results, and two methodological differences may help explain this disparity: first, Bendall and Restall (1983) used a buffer at pH 5.5 to measure length changes and also demonstrated that the volume changes were less dramatic when cooking occurred at pH 6.8. Our study used pH 7, potentially demonstrating a pH effect on heat-induced denaturation of myofibrils. The second difference relates to the possible influence of storage on myofibril integrity. Bendall and Restall (1983) held the samples for 24 h after slaughter at 10°C, sufficient time to allow some level of proteolytic degradation of myofibrils, whereas the fibre preparation used here was immersed in protease inhibitors immediately after slaughter. Myofibrillar proteins may have undergone sufficient proteolysis to have influenced their response to heating. Chapter 3 shows that tension development in skinned ovine muscle fibres treated with trypsin is significantly lower during heating than intact controls. This suggests that a myofibrillar component contributing to tension onset during heating

and the magnitude of isometric tension development, depends on the myofibrillar proteins remaining intact. The degree of reduction in fibre diameter, however, is not influenced by proteolysis during storage.

In summary, I report here the contribution of sarcomere length to the stiffness of single skeletal muscle fibres in the uncooked and cooked states. The methodology described here identifies the length of the I-band as a potentially key myofibrillar structure defining the initial tenderness of cooked meat. More circumstantial evidence identified an important role for titin in fibre stiffness and its contribution to tension changes during the thermal denaturation of the thin and thick filaments. Further research is required to establish if statistical differences in stiffness, especially in the uncooked state, can be found. Visual trends indicated the presence of differences in the sarcomere length related stiffness, but the standard deviations in this study were high and a greater number of fibres were needed to increase the power of the analysis.

Further study is needed to evaluate the level of titin contribution at sarcomere lengths in excess of 2.5 μm and to the mechanism of titin denaturation during heating.

Chapter 3

The Effect Of Selective Titin Degradation On The Structural Characteristics Of Ovine Muscle Fibres

3.1 Introduction

The increase in tenderness in meat during post-mortem ageing is recognised as a highly variable process, in both its rate and its extent, a fact that has considerable commercial implications. The increase in tenderness is attributed to the proteolytic degradation of myofibrillar structural proteins, as identified by SDS PAGE (Bechtel and Parrish, 1983; Xiong and Anglemier, 1989; Huff-Lonergan *et al.*, 1996b). A number of proteins can be shown to degrade gradually, while putative degradation products appear. The loss of structural integrity means that meat will have a lower yield point.

The reasons for variability in the ageing process are not well understood. The variable activities of the principle enzymes involved in protein degradation in post-mortem meat, the calpains, have been implicated, particularly through differences in the concentration of their specific inhibitor, calpastatin (Geesink and Koohmaraie, 1999; Goll *et al.*, 2003). However, changes in tenderness during post-mortem ageing could also be understood by identifying the key structural proteins whose degradation contributes to tenderness in meat.

Under tensile load, where fibres are stretched along the longitudinal axis, the yield point of the muscle fibre structures appears reproducible: when examined microscopically, the break of the myofibrils is typically in the I-band, near to, but not within, the Z line (Locker and Leet, 1975; Locker and Wild, 1982). The actin

and myosin that constitute the major proportion of structural proteins do not appear to degrade during post-mortem storage except when stored at temperatures in excess of 20°C (Bechtel and Parrish, 1983; Sancho *et al.*, 1997). The general concept of how degradation might contribute to tenderness can be considered in terms of the axial and radial components of muscle structure. The main axially-aligned structural proteins are titin, nebulin and filamin, and their orientation provides an obvious mechanism for how structural integrity is maintained when meat is placed under mechanical load in parallel with fibre direction (Davey and Dickson, 1970; Locker and Wild, 1982). An often observed dissolution of the Z-band (Davey and Gilbert, 1967), attributed to the release, though not degradation, of α-actinin (Taylor *et al.*, 1995), may also contribute to tenderisation, although most of the histological evidence suggests that the thin filaments break within the I-band rather than at the connection of the thin filament with the Z-line (Davey and Dickson, 1970). In addition, the radial elements undoubtedly contribute to the structural integrity of both muscle and meat: by distribution of the load, weak areas can be bypassed (Lieber *et al.*, 2001).

The variation in tenderness that occurs between muscles can, in part, be attributed to extent and composition of the extracellular matrix, particularly the collagen component (Light *et al.*, 1985; Christensen *et al.*, 2000). Cell size and type are also likely to have some effect, both in terms of the enzyme activity and the structural proteins (Maltin *et al.*, 2003). In addition to the differing rate of activity of myosin ATPase and the various myosin isoforms in fast and slow-twitch fibres, Ouali and Talmant (1990) demonstrated that calpastatin concentration differs between fast and slow type muscle fibres and that conditioning is negatively correlated with this concentration. This suggests that if calpastatin levels are highest in slow-twitch red

muscles and lowest in fast-twitch white muscles, then conditioning favours fast-twitch white muscles.

A better understanding of the relative roles of specific structural proteins would help to understand variability. Titin is in many respects a likely candidate protein, a possibility first proposed by Locker (1977) after his discovery of gap filaments, now referred to as titin.

Maruyama *et al.* (1977) were the first to isolate the primary elastic myofibrillar protein, titin (or connectin). These authors isolated a total of 5.5% (w/w) of titin from pure myofibrils. This was later increased to 10% (w/w) through modifications to the extraction technique (Wang *et al.*, 1979). Titin is a large protein (3,000 kDa; Trinick 1992) consisting of two super-repeat motifs; an immunoglobulin domain (I-set), and type III fibronectin domains (Trinick 1996). Titin also has an unique sequence in the I band region termed the PEVK domain, so named due to a localised increase in the proline (P), glutamic acid (E), valine (V), and lysine (K) amino acids (Labeit and Kolmerer, 1995; Maruyama 1997). The titin filament is in excess of 1 μm long, and the PEVK domain is thought to be responsible for much of its elastic properties (Linke 1998). Titin fibrils extend from the Z-line to the M-line providing elastic links within the sarcomere. It also plays an important role in regulating thick filament assembly during muscle development and contraction (Labeit and Kolmerer, 1995; Kellermayer and Granzier, 1998).

Titin can be selectively removed from a single muscle cell by brief exposure to low concentrations of trypsin, an effect attributed to its large molecular weight. Yoshioka *et al.* (1986) and Higuchi (1992) selectively degraded titin in single, mechanically skinned, frog m. *semitendinosus* fibres. There was no obvious

evidence of hydrolysis of other proteins as judged by SDS-PAGE and electron microscopy, although a slight density change was evident in the Z line. Maruyama and colleagues (1977) found that the elastic properties of skinned fibres decreased after proteolytic treatment and that they were easily broken during stretch. Untreated fibres, although not as easily broken, displayed an irreversible hysteresis when the fibre was stretched to 140% of its resting length suggesting that irreversible damage occurred during the stretch. This implies that the structure or structures affected by proteolysis in the treated fibre are preventing the untreated fibre from breaking even after the damage caused by tensile strain.

The ability to selectively degrade a potentially important structural protein in muscle fibres offers the opportunity to investigate the contribution of this protein to the response to strain of isolated single fibres and, potentially, to meat tenderness. This study reports on the effects of selective titin degradation on the structural characteristics of single ovine muscle fibres in both the native and cooked states.

3.2 Materials and Methods

Fibre preparation, experimental set-up, sarcomere length measurement, fibre typing, and heating procedure, were all carried out as described in Section 2.2.

3.2.1 Stiffness Measurements

Fibre stiffness was measured by imposed sinusoidal oscillations as described in Section 2.2.6, with some modifications to accommodate the fragility of fibres after trypsin digestion. A baseline tension of 3.3 kN/m^2 was applied before imposing oscillations of 5 nm/h.s, at a frequency of 5 Hz. These same conditions were used for both uncooked and cooked fibres. All measurements were made at room

temperature (20°C). Fibre stiffness in response to applied oscillations was calculated as described in Section 2.2.6.

In addition to the stiffness measurements, fibres were subjected to a continuous stretch to ~15-20% of their resting length, using a stretch rate of 73 μm/s.

3.2.2 Trypsin digestion

Preliminary experiments were carried out to determine the concentration of trypsin and duration of exposure needed to produce selective degradation of titin in ovine m. *psoas major* fibres. Higher concentrations of trypsin, and longer durations of exposure, were needed to produce a level of degradation (observed using SDS-PAGE; Figure 3.1), comparable to those reported for frog (Higuchi 1987; 1992; Yoshioka *et al.*, 1986), chicken (Maruyama *et al.*, 1977), and rabbit (Funatsu *et al.*, 1990).

All fibres were set at a sarcomere length of 2.1 μm, and the fibre length and diameter were recorded. The fibre was placed in a trough of rigor solution and an initial measure of fibre stiffness was made. Trypsin was then added to the trough to a final concentration of 2 μg/ml and a number of the fibres were subjected to proteolytic degradation for 10 min (n = 6). Fibre stiffness was recorded at 2 min intervals during exposure to trypsin. A drop in fibre stiffness during this period was interpreted as evidence of proteolytic damage to actomyosin and the fibre was discarded (Higuchi 1987). A number of fibres were left to dwell in RigS for 10 min in the absence of trypsin and these acted as the control fibres (n = 8).

On completion of the trypsin digestion, soybean trypsin inhibitor was added in excess (~100 μg/ml), and the stress response of the fibre was once again recorded. The fibre was then heated to 80°C at a rate of 9.2°C/min. The fibre was maintained

at 80°C for 3.5 min before being cooled to 20°C. The stress response to the 5 Hz oscillation was recorded and then, irrespective of length, the fibre was stretched by 458 μm at a constant rate of 73 μm/s.

3.2.3 Gel electrophoresis

This was carried out as described in Section 2.2.5.1.

3.3 Results

In this study the pancreatic serine protease trypsin was used to selectively degrade titin so the effects of the degradation of one protein could be analysed.

Using SDS-PAGE, it was determined that 2 μg/ml of trypsin for 10 min produced significant degradation of native titin (T1; 3,000 kDa) to the lower molecular weight product (T2; 2,400 kDa), without evidence of myosin or nebulin degradation products in single fibres (Figure 3.1). More prolonged exposure to trypsin (1.25 μg/ml) was experimentally assessed using a purified myofibrillar preparation. Although the trypsin concentration was slightly lower, the surface area of protein exposed and the duration of exposure was greater. There was clear evidence of proteolytic products forming immediately below myosin after as little as 10 min, however, nebulin appeared to remain intact for up to 60 min (Figure 3.2).

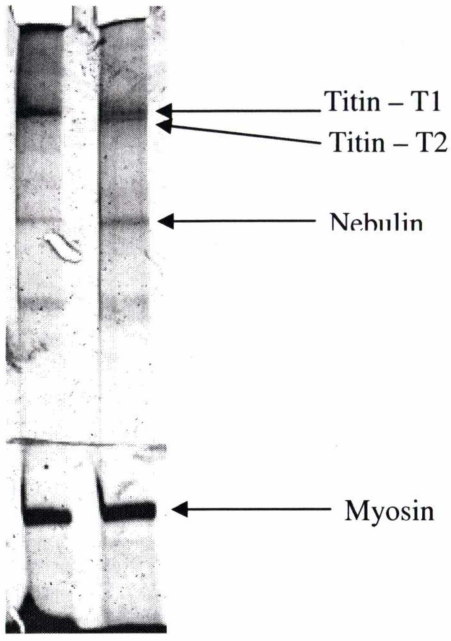

Figure 3.1 Electrophoretogram of single muscle fibres exposed to 2 µg/ml of trypsin. Lane 1=Control, and Lane 2=10 min trypsin exposure. T1=intact titin, T2=2,400 kDa titin breakdown product. Myosin bands were identified using a molecular weight marker.

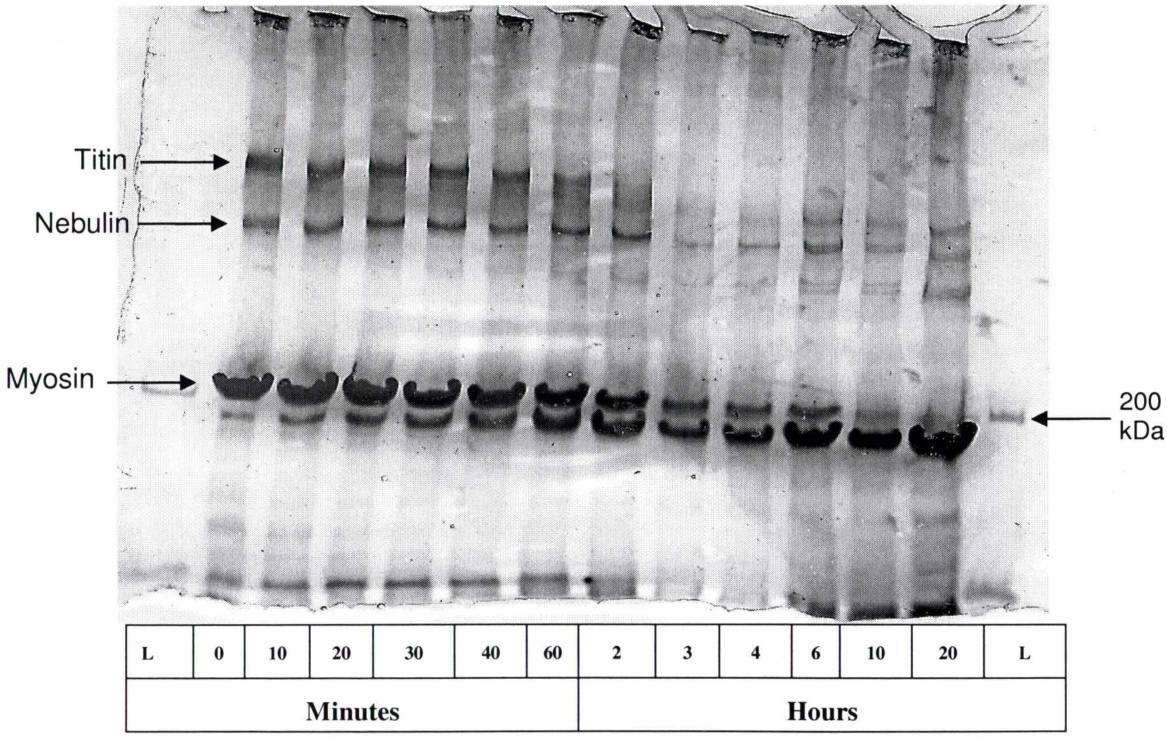

Figure 3.2 Time dependent trypsin digestion of a purified myofibril preparation – 4% Gel (100:1). Prolonged exposure to trypsin indicates greater degradation of both nebulin and myosin.

There was no significant effect of trypsin on the stiffness of raw fibres (P=0.185). However, after cooking, a significant ($P \leq 0.05$) reduction in stiffness was evident in the trypsin-digested fibres (stress/half sarcomere = 1.0 N/m^2; SEM = 0.1; n = 6) when compared to cooked control fibres (stress/half sarcomere = 3.5 N/m^2; SEM = 0.6; n = 8; Figure 3.3). Note that the result in this Chapter for control fibre stiffness differs from the value of 6.9 kN/m^2 given in Chapter 2. This is due to differing initial baseline tensions imposed on fibres prior to stiffness measurements. The baseline tension originally used in Chapter 2 had to be reduced for this study as the trypsin treated fibres could not withstand that level of initial tension.

Trypsin treatment also reduced the extensibility of cooked muscle fibres. While no control fibres broke during the stretch (between 0 and ~15% of rest length), all treated fibres broke at an average of 7.6% of resting length (SEM = 1.2, n = 6; see Figure 3.4).

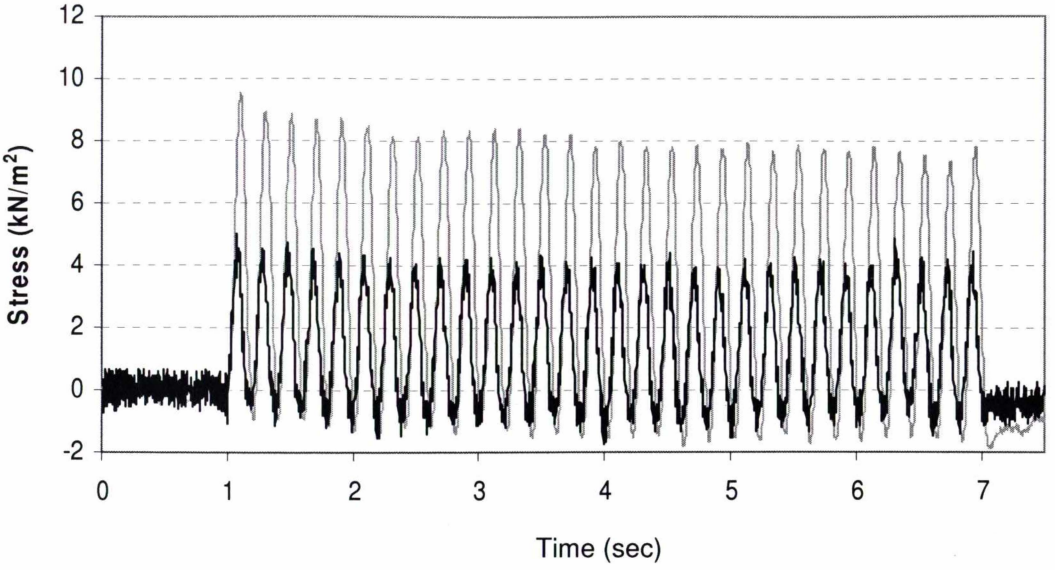

Figure 3.3 Response of two separate cooked fibres to a 5 Hz sine wave oscillation after heating to 80°C. Control (grey), trypsin treated prior to cooking (black). Sarcomere length = 2.1μm.

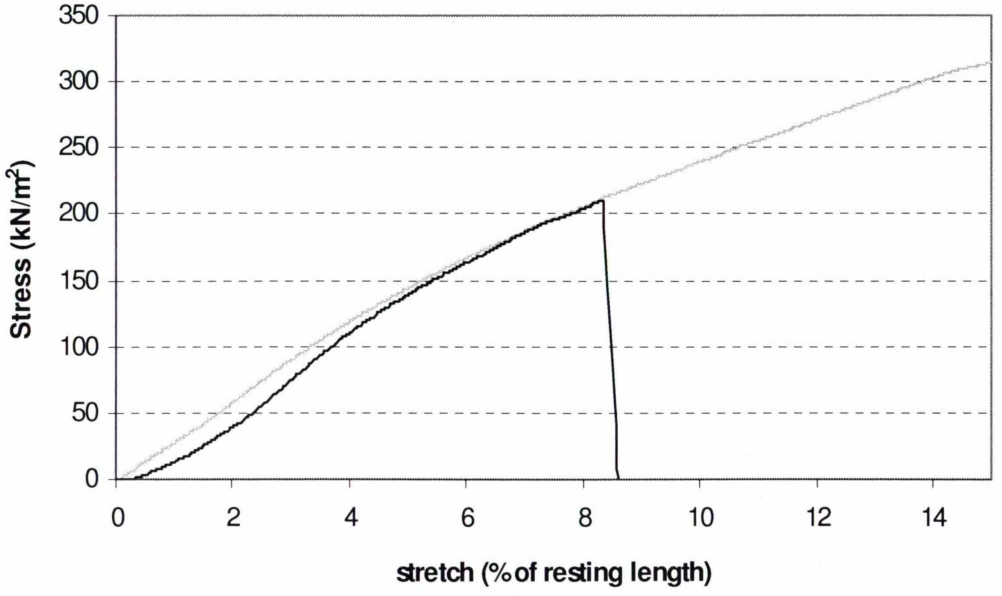

Figure 3.4 Example of stretching two cooked fibres to ~15% of resting length (fibre length normalised to 3 mm). Control (grey), and trypsin digested (black). The response of the trypsin-treated fibre has been normalized to that of the control.

3.4 Discussion

Trypsin hydrolyses titin from T1 (~3,000kDa) into T2 (~2,400kDa). A third titin degradation product is also evident; this has been tentatively identified as 1,200 kDa (Matsuura *et al.*, 1991; Huff-Lonergan *et al.*, 1996; Suzuki *et al.*, 1996). The trypsin proteolysis appears to have been selective to titin as no breakdown products of myosin were evident on the gel. Nebulin was not consistently resolved on single fibre gels but appeared intact following myofibrillar digestions. The stiffness of the fibre after 10 min exposure to 2 μg/ml of trypsin was equivalent to the pre-digestion stiffness.

The proteolysis of titin in the raw state did not significantly affect the stiffness of the fibre. The greatest compliance of individual skinned fibres in the raw state is attributable primarily to actomyosin cross-bridges (Linari *et al.*, 1998), a conclusion confirmed in Chapter 2 using the present methodology. By implication, the remaining structures transferring the applied strain, in particular the thick and thin filaments and Z-lines, must be considerably less compliant than the cross-bridges. Since titin degradation did not affect stiffness in the raw fibres, it can be concluded that titin does not contribute to the compliance of any participating structures.

However, after cooking to 80°C, there was a reduction in the stiffness of the trypsin digested fibres. Chapter 2 discusses the correlation between the increase in the stiffness of cooked fibres with proportional decreases in sarcomere length and concluded that the stiffness of cooked fibres is a function of the length of the I-band. That the stiffness of cooked fibres should be substantially reduced by prior degradation of titin therefore suggests that an interaction exists between titin and I-band compliance during or after the cooking process. This is not an unreasonable

expectation on the basis of the organisation of these sarcomeric structures. This study suggests that when titin remains intact it interacts with the thick and thin filaments directly before, or during, thermal denaturation, imposing additional strength and rigidity to the denatured structure. When titin is degraded by proteolytic activity, the interaction is compromised and the entire protein structure is weakened.

Mutungi *et al.* (1996) reported that aging of meat led to a reduction in both the breaking strain and stress of single fibres. This is also demonstrated in Figure 3.4 after the single fibres were exposed to 2 μg/ml of trypsin. A reduction in the breaking strain and stress of fibres reflects an increase in tenderness of whole meat (Mutungi *et al.*, 1995).

Titin and actin undergo thermal denaturation at an average temperature of 75-80°C (Pospiech *et al.*, 2002). However, titin requires less energy to begin to denature (lower heat of transition) than actin and a lower initial denaturation temperature but a similar temperature maximum results in a far broader thermograph peak. Titin can also be degraded into smaller polypeptide fragments followed by partial aggregation (Fritz *et al.*, 1992). It is thought that titin may be partially responsible for meat toughening when muscle is heated above 60°C (Pospiech *et al.*, 2002). It is not clear whether heating is responsible for the degradation of titin or whether proteolytic events are wholly or partially involved, as suggested by King *et al.* (1981). Considering the findings described in this chapter, I postulate that titin is thermally denatured, and that this denaturation occurs in association with an interaction between titin and actin. This protein-protein interaction may occur as a result of titin unfolding during denaturation coupled with changes in the conformation of the thin filament. For example, in smooth muscle, the unfolding of tropomyosin at ~45°C is

accompanied by its dissociation from F-actin (Levitsky *et al.*, 2000). If titin associates with actin in the early stages of thermal denaturation to result in a stronger, more elastic I-band, then a proteolytic break in titin prior to this association could prevent this process and reduce the axial strength of the I-band.

Further work needs to be done to investigate the impact of various sarcomere lengths, together with proteolytic digestion, on the stress response of ovine single muscle fibres, both before and after thermal denaturation.

Chapter 4

The temperature dependence of the stiffness of ovine m. *psoas major* fibres before and after thermal denaturation of myofibrillar proteins

4.1 Introduction

4.1.1 Meat as a cooked product

Conventionally, meat products are aged and cooked before being consumed. These two processes are of primary importance to the eating quality of meat. As a general rule, tenderness is enhanced by postmortem storage (Davey and Gilbert, 1967) but decreases during heating to 80°C. This cooking-induced decrease in tenderness occurs in 2 phases: the first occurs below 60°C and appears to be related to changes in the mechanical properties of perimysial connective tissue; and the second phase of toughening occurs above 60°C and correlates with an increase in the breaking strength of single muscle fibres (Christensen *et al.*, 2000).

The change in texture of meat as it is cooked is a direct result of the denaturation and subsequent gelation of myosin and actomyosin. Denaturation occurs when the tertiary structure of a protein becomes disordered due to unfolding, but involves no alteration to the protein's primary structure. This process of unfolding is believed to involve different parts of the protein denaturing at different rates (Ziegler and Acton, 1984).

4.1.2 Myofibrillar transitions – gelation

Hermansson (1977) defined the term 'aggregation' as collective protein-protein interaction during denaturation. The difference between coagulation and gelation is that coagulation is a random aggregation while gelation is the formation of continuous protein structures during denaturation. Gelation involves the formation of an ordered, three dimensional network system of proteins linked by hydrogen bonds. If this hydrogen-bonded system is allowed to form slowly, the gel can reach high levels of elasticity as the chains are given greater opportunity to orient themselves. For this process to be successful, denaturation must occur before aggregation (Ziegler and Acton, 1984).

The structural properties of cooked muscle tissue are remarkably different compared to raw meat. Factors such as sarcomere length, aging, and connective tissue content contribute to the final product. In addition, the gel that forms as the proteins denature is itself influenced by the meat pH and final temperature (O'Neill *et al.*, 1993). Many of the effects of cooking on the properties of whole meats have been studied in purified actomyosin systems (Kimura *et al.*, 1980; Yasui *et al.*, 1980; Acton *et al.*, 1981). The mechanical properties of actomyosin gels are greatly affected by protein concentration, pH and heating temperature. Protein concentration and final temperature increase gel strength, while an increase in pH results in a decrease. Gel strength is considered to represent the degree of intermolecular cross-linking; increasing the temperature increases the gel strength through a greater degree of cross-linking (O'Neill *et al.*, 1993).

4.1.3 Myosin denaturation

Myosin is termed a 'hybrid' protein as it is comprised of two helically interwoven polypeptide strands, each with a globular head (Ziegler and Acton, 1984). During heating *in vitro*, myosin heads initially aggregate at a temperature of 48.5°C (pH 6.0), and this is followed at 57.5°C by the unfolding of the myosin tail to form a cross-linked network (O'Neill *et al.*, 1993). These processes are responsible for much of the physical change that accompanies cooking. Myosin filaments are shorter and thinner after heat treatment but the actin binding ability of myosin heads remains (Kimura *et al.*, 1980).

The transition temperature is defined as the temperature at which the native protein begins to denature (Wright *et al.*, 1977). Differential scanning calorimetry (DSC) detected either two or three transition temperatures for isolated actomyosin depending on the ionic strength of the environment. The ionic environment and pH influence the transition temperatures of isolated myosin and, at a pH of 7 and an ionic strength of 0.046 M, there appeared to be only one, at 55°C (Ziegler and Acton, 1984). At a higher ionic strength (~1 M) the first transition is thought to involve the denaturation and aggregation of the myosin heads, the second transition is due to the unfolding of the myosin tail, while the third is attributed to the denaturation of actin.

4.1.4 Effect of sarcomere length on single muscle fibres

Willems and Purslow (1996) demonstrated that uncooked cold-shortened, control and stretched single muscle fibres showed no significant differences in maximum stress at fibre yield point, whereas the maximum stress of thermally denatured cold-shortened fibres was significantly greater than both the control and stretched muscle

fibres. This confirms that differences in sarcomere length affect single fibres in the same way as whole meat. This obvious increase in maximal stress of cold-shortened muscle fibres could be due to the masking or a change in location of the so-called 'weak' point located in the I band (Marsh and Carse, 1974). In addition to this, or alternatively, increased overlap of the contractile filaments could result in a fundamentally stronger myofibril (Willems and Purslow, 1996).

4.1.5 The temperature dependence of meat stiffness

There are many temperature-dependent changes in raw whole meat as the heating temperature increases; fibre diameter and sarcomere length decrease around 60°C, the loss of water increases as temperature increases, toughening occurs between 40 and 50°C, a further increase in toughness occurs between 65 and 75°C, and a third increase between 70 and 90°C (Davey and Gilbert, 1974; Bouton *et al.*, 1981; Palka and Daun, 1999).

While the temperature-associated changes of whole meat and single muscle fibres during heating are well documented, changes in the mechanical properties of muscle fibres following heating to 80°C has not been reported. The temperature dependence of the stiffness of fibres that have already been heated to 80°C became evident during experimentation into final cooking temperature. Initially this study was to examine the stiffness of single fibres at varying final temperatures. However, during data analysis, significant discrepancies between results reported at 80°C arose between the current results and those reported in Chapter 2. In Chapter 2, sarcomere lengths of 1.6 μm showed very similar stiffness responses for both cooked and uncooked fibres, whereas the stiffness response from cooked fibres with a sarcomere length of 2.1 μm were statistically lower than uncooked fibres at the same sarcomere

length. At first glance, the current results indicated that there was an obvious reduction in the stiffness of fibres at a sarcomere length of 1.6 μm as the temperature was increased from 7 to 80°C. This was in direct contradiction to results given in Chapter 2 and further investigation revealed that the source of this difference lay in the temperature at which the analysis of stiffness was done.

This study therefore aimed to investigate the temperature dependence of stiffness in raw and thermally denatured single fibres.

4.2 Methodology

Single *psoas major* muscle fibres were held at 2 sarcomere lengths, 1.6μm, and 2.1μm and were heated in-vitro from 7°C to specific end-point temperatures; 20, 40, 50, 60, 70, 75, and 80°C. At each of these temperatures, stiffness was measured to define temperature-dependent behaviour.

Following heat denaturation, the bath temperatures were reduced, in a stepwise manner from 80°C to 70, 60, 50, 40 and finally, 20°C. At each temperature, stiffness measurements were repeated.

Fibre preparation, experimental set-up, sarcomere length measurement, and fibre typing, were all carried out as described in Section 2.2.

4.2.1 Heating procedure

The heating procedure was carried out essentially as described in Section 2.2.7, with some modification. At a rate of 1°C/s, the water bath temperature was increased from 7°C to temperatures of 20, 40, 50, 60, 70, and 80°C. Each temperature was held constant for 60 s to allow the fibre temperature to fully equilibrate. After the 60 s dwell time, the fibre stiffness was measured at the respective temperature.

Once the fibre stiffness had been recorded at 80°C, the bath temperature was then reduced to 70, 60, 50, 40, 20 and finally 7°C. As before, the temperature was held for 60 s to allow the temperature of the fibre to equilibrate before stiffness was measured.

4.2.2 Statistical Analysis

Raw data were analysed using the 2 way ANOVA General Linear Model with ad-hoc pairwise comparisons (Tukey). Data were then normalised to a temperature of 7°C (raw fibre) for each sarcomere length for graphical display (see Figure 4.1 and Figure 4.2).

4.3 Results

4.3.1 Temperature dependence of fibres at sarcomere lengths of 1.6 μm

As the temperature increased from 7 to 50°C no significant drop in stiffness was detected (Figure 4.1), however, the stiffness of the fibre at 20°C was ~20% less than the stiffness at 7°C. The significant reduction in stiffness occurred between 40 and 70°C, with no further measurable changes in stiffness at higher temperatures.

A very definite temperature dependence in fiber stiffness was evident for fibres with sarcomere lengths of 1.6 μm and heated to 80°C (Figure 4.1). From 80-60°C there was very little difference in fibre stiffness response but, from 60-40°C, fibre stiffness increased almost 30%. At 7°C, the thermally denatured fibres were significantly stiffer than fibres measured at temperatures >50°C ($P < 0.05$) and had a stiffness equivalent to that of the raw state. These results are consistent with those given in Chapter 2.

4.3.2 Temperature dependence of fibres at sarcomere lengths of 2.1μm

As the temperature increased from 7 to 50°C no significant drop in stiffness was detected, however, fibres with a 2.1 μm sarcomere length displayed a 20% reduction in stiffness between 7 and 20°C, a trend also seen in fibres with shorter sarcomeres. Once the fibre was heated past 50°C there was a significant reduction in stiffness ($P < 0.05$); over half of the total reduction (~60%) in stiffness occurred between 50 and 60°C. A further reduction in stiffness of approximately 15% occurred between 60 and 70°C with no apparent change evident at greater temperatures.

Fibres with longer sarcomere lengths showed a very different response to cooling when compared to fibres with shorter sarcomere lengths (compare Figure 4.1 and Figure 4.2). Cooling fibres with a sarcomere length of 1.6 μm resulted in a full recovery of stiffness at all temperatures whereas fibres with a sarcomere length of 2.1 μm showed no stiffness recovery upon cooling. These results are also consistent with those given in Chapter 2.

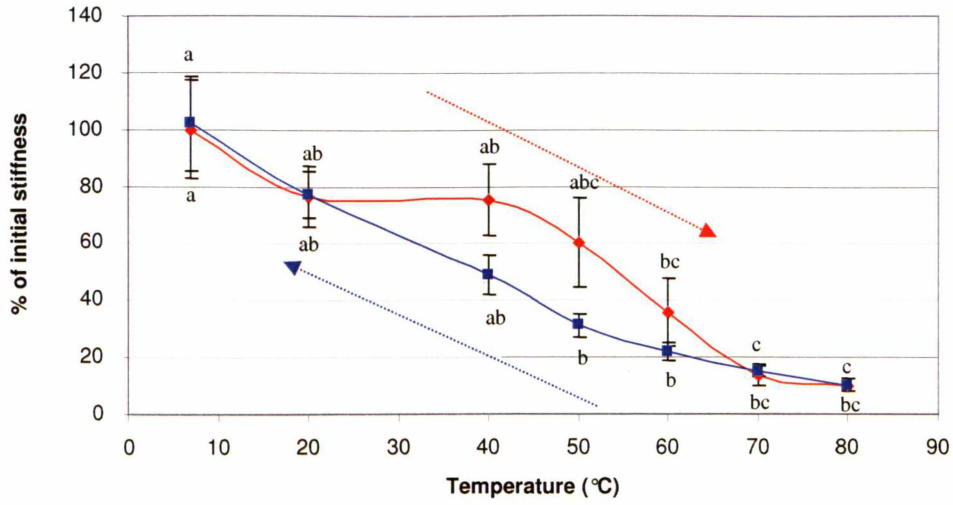

Figure 4.1 The temperature dependence of ovine m. *psoas major* fibre stiffness at shortened sarcomere length (1.6 μm) as fibres were heated to 80°C (red; n=10) and then cooled back to 7°C (blue; n=4). Different letters indicate a significant difference in stiffness during heating and cooling and also between like temperatures (P<0.05). Arrows depict progression of time. Data has been normalised to stiffness of raw fibres at 7°C.

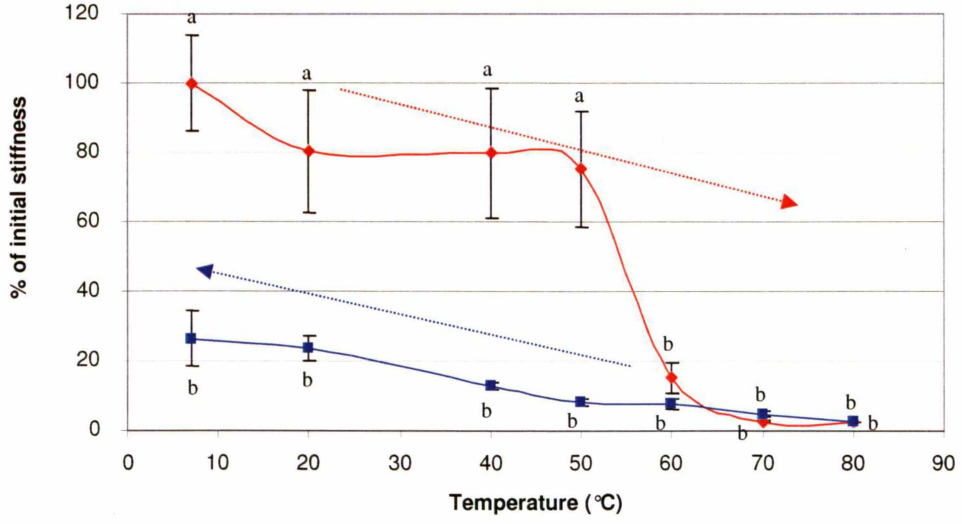

Figure 4.2 The temperature dependence of ovine m. *psoas major* fibre stiffness at resting sarcomere length (2.1μm) as fibres were heated to 80°C (red; n=10), and then cooled back to 7°C (blue; n=4). Different letters indicate a significant difference in stiffness during heating and cooling and also between like temperatures (P<0.05). Arrows depict progression of time. Data has been normalised to stiffness of raw fibres at 7°C.

4.4 Discussion

A surprising similarity in fibre stiffness was seen between the short (1.6 μm) and resting (2.1 μm) sarcomere lengths at 80°C (see Figure 4.1 and Figure 4.2). This similarity at first glance does not appear to agree with the results given in Chapter 2 (see Figure 2.11), but further exploration indicates that these results are, in fact, fully consistent. As shown in Chapter 2, heating fibres of varying sarcomere lengths to 80°C results in varying stiffness responses when measured at 7°C. Under these conditions, fibres with short sarcomere lengths (1.6 μm) appear to have increased resistance to deformation, suggesting that denaturation results in stronger material properties (Willems and Purslow, 1996).

Clearly, the stiffness of cooked fibres, and raw fibres for that matter, depends on the temperature at which the stiffness is measured. It is previously documented that rigor fibres that undergo an abrupt change in temperature (or T-jump) display a reduction in stiffness due to the thermoelasticity of the cross-bridges and thermal expansion of the myofibrillar proteins (Bershitsky and Tsaturyan, 1989). For this reason it was not a surprise to observe a 20% decrease in stiffness at both sarcomere lengths in the raw fibres between 7 and 20°C. However, temperature appears to play an important role even after thermal denaturation and gelation of myofibrillar proteins has taken place, an unexpected effect that has not previously been reported. Gel matrices formed at shorter sarcomere lengths are more sensitive to subsequent changes in temperature. In general, this temperature dependence will be a function of the stability of the gel – cooler temperatures produce a more stable gel matrix. However, the reason for the sarcomere length effect is more difficult to explain. The stability of this matrix at varying temperatures would be most evident if the gel was

continuous and not interrupted by 'weak' I bands – as in fibres with sarcomere lengths of 1.6 μm. Sarcomere lengths greater than 1.6 μm lack myosin filaments that may be butting up against or puncturing the z-line, causing continuous and strong thick filament structures upon heat denaturation; at 2.1 μm, the fibre still maintains 100% cross bridge overlap but an I band has formed (an I band will form at all sarcomere lengths longer than 1.6 μm). The formation of this I band requires actin and/or titin to provide the continuity throughout the gel matrix. As the sarcomere length increases further, a lengthening I band would contribute increasing compliance in the protein structure of the gel, and, at temperatures of 50°C or greater, the actin and/or titin would be responsible for linking the denaturing myosin filaments (Bouton *et al.*, 1981).

This poses some interesting questions with regard to the toughness of cold shortened whole meat. If the stiffness of heat-denatured myosin is temperature-dependent, how does that relate to the temperature dependence of texture in whole meat? Whole meat samples are normally instrumentally tested at temperatures of 7°C or less for textural and shear characteristics. It is recognized that the toughness caused by cold shortening appears only after cooking (Bouton *et al.*, 1981); is this toughness also dependant on the temperature at which it is analysed or eaten? Further work needs to be done in this area to demonstrate whether these observations translate into obvious differences in the toughness or tenderness of whole meat at various temperatures.

Chapter 5

General Discussion

This study aimed to characterize the mechanical properties of ovine single skeletal muscle cells before and after heating to 80°C. A technique was developed to study the tensile characteristics of individual skeletal muscle cells, both in the uncooked and cooked states. The study of single fibres offers a number of important advantages compared to studies using whole tissue. Whole tissue includes a range of fibre types and fibre sizes, and includes a range of structural components, in particular the myofibrils and extracellular matrix. Analysis of single fibres allows the myofibrillar contribution to meat tenderness to be assessed in isolation, and offers the potential to discriminate between the contributions of specific structural proteins that make up the myofibrils.

The development of the single fibre technique enabled the study of changes in mechanical responses in muscle fibres that accompanied a variety of experimental manipulations. These manipulations are similar to those that muscles and meat undergo in the postmortem period.

Muscle contraction and the resulting sarcomere length are extremely important to the quality of meat. To better understand how different sarcomere lengths affect single fibre stiffness (resistance to small amplitude strains), the properties of individual fibres at different sarcomere lengths was examined. The stiffness of raw fibres was equivalent at lengths of 1.8 and 2.1μm, but was appeared reduced at the longer sarcomere length of 3.8 μm. The stiffness of the raw fibres is therefore inferred to be a function of the number of bound cross-bridges.

When whole meat is heated to temperatures in excess of 50°C, shortened sarcomere lengths correlate to increases in toughness as a result of the increase in the strength of the denatured and aggregated myofibrillar proteins. The properties of a single fibre at various sarcomere lengths after cooking did follow a similar pattern to that of whole tissue after cooking. Reduced sarcomere length in single fibres demonstrated an increased resistance to extension (stiffness). Also, an increase in isometric tension was measured in single fibres as myofibrillar proteins denature, consistent with reports from whole tissues. To the extent that the stiffness of isolated muscle fibres follows a similar pattern to the tenderness measurements of whole meat, the methodology would appear to provide a novel experimental methodology for understanding the structural determinants of meat tenderness.

Proteolysis is a key event in the aging process, and the effect that this event has on protein structure of the muscle cell determines how tender the meat becomes (Davey and Gilbert, 1967). A key structural protein that contributes to maintaining the organisation of living muscle under strain is titin. Titin connects the thick filament to the Z-band and, through its remarkable elastic properties, plays a major role in holding the thick filament in alignment with the thin filaments in the sarcomere. Titin has also been identified as possibly contributing to toughness in meat, and whose degradation by proteolytic enzymes, particularly calpain, contributes to tenderisation. However, calpain-induced proteolysis degrades a range of myofibrillar structural proteins and it is generally assumed that tenderness is the product of a non-specific process, the cumulative effects of a range of structures. A methodology has been described that allows titin to be selectively degraded in single fibres by appropriate treatment with trypsin (Maruyama *et al.*, 1977), and created the opportunity to analyse the contribution to toughness this protein makes in cooked

fibres. Selective removal of titin by trypsin weakened the fibre only after cooking, and fibres broke at significantly lower strains. This result strongly suggests that titin plays a critically important role in the development of tenderness in meat.

The act of cooking produces a wide range of structural changes as proteins denature. An important consequence is the formation of an actomyosin gel, the structural properties of which are defined by the immediate environment in which the gel forms (Acton *et al.*, 1981; O'Neill *et al.*, 1993). Chapter 2 discusses the impact of sarcomere length on the structural properties of the gel and the increasing elasticity of the I-band as fibres are heated at increasing sarcomere lengths. In raw fibres, compliance is primarily provided by the cross-bridges and stiffness is proportional to the total number of bound cross-bridges (Higuchi *et al.*, 1995). Once fibres have been heated to 80°C and the proteins have been denatured, the compliance exists almost entirely in the thin filament and thin filament compliance is greatly increased by heating.

In Chapter 4 the temperature-dependence of thermally denatured myofibrils at short (1.6 μm), and resting sarcomere length (2.1 μm) was examined. A surprising difference in the stiffness response of heat denatured fibres at two different sarcomere lengths raises some questions about how this temperature dependence affects pre-cooked whole meat products and its commercial implications.

The experiments described in this Thesis provide a new methodology to understand the stiffness of single muscle fibres, and has resulted in a better understanding of the contribution of myofibrillar proteins to muscle cell integrity during aging and cooking. Further work is required to better characterise the denaturation of titin, and the protein-protein interactions with both actin and myosin during heating. For

instance, how does titin contribute structurally to gel strength during and after cooking, and how does proteolysis prior to cooking affect this contribution? The temperature-dependence of the stiffness of cooked single muscle fibres, especially at short sarcomere lengths (1.6 μm), was a surprise and not previously reported. Further work needs to be done to evaluate the protein interactions and biochemical properties of actomyosin gels as temperature is reduced, and establish what, if any, effect this has on pre-cooked meat products. Sarcomere length, proteolysis and cooking all affect the functionality of a single muscle cell, however, the correlation of these changes in single fibres to meat as an edible product, also requires further investigation.

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