Quantification of the Calpain Proteolytic System, and the effects of Growth Hormone enhancer and Selection for glucose tolerance on the Calpain Proteolytic System and tenderness in Lambs.

A thesis submitted in partial fulfillment of
the requirements for the degree
of
Master of Science
at
Lincoln University

by

Matthew Peter Kent

Lincoln University
1994

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The calpain proteolytic system has been implicated in determining the tenderness of post-mortem muscle. An appreciation of factors that impact upon this system may allow commercial meat producers and processors to generate meat of greater tenderness. This thesis examines the effect of glucose tolerance and growth hormone enhancer on the activity of enzymes belonging to the calpain system, with a view to developing a better understanding of the system.

A variety of ion exchange and hydrophobic chromatography methods used to extract calpains were compared. DEAE based methods of extraction were superior to phenyl sepharose with respect to accuracy and reproducibility of calpain separation. A protocol was developed and used in subsequent experiments.

Previously sheep had been bred selectively based upon their rates of glucose clearance. The two lines produced, displayed fast and slow clearance and also presented differing body compositions. Fast sheep generally had lower % protein but higher % fat compared to slow ($p<0.05$). Fast sheep also had significantly greater ($p<0.05$) activity of calpain I and II extracted from post-mortem skeletal muscle, and lower calpastatin. This may indicate that the lower % protein was attributable to augmented proteolytic enzyme levels. Enzyme levels were not correlated with differences in tenderness or pH of meat from these animals.

Immunogenic peptide fragments of growth hormone can associate with antibodies and

generate an amplified GH response. Experiments were performed to confirm this by injecting lambs with a GH peptide. No effect on growth rate was observed, although an immune response did occur. This treatment had no effect on body composition or calpain activity, and no differences in tenderness were observed.

This work succeeded in developing a reliable protocol for the extraction and quantification of the calpain system. Heightened protease levels were correlated with lower protein content in sheep selected for fast clearance of glucose. This may indicate a function for the calpains in *in vivo* nutrient partitioning. The GH enhancing peptide used in this work failed to produce a change in the hormonal status of the animals, and had no effect on meat quality.

Keywords: Calpain, calpastatin, sheep, chromatography, glucose tolerance, growth hormone enhancer, meat tenderness.

Acknowledgements

This thesis was made possible with the assistance and support of many people to whom I owe a debt.

I would like to sincerely thank my supervisor Dr. Roy Bickerstaffe who has provided assistance, encouragement, personal commitment and enthusiasm for the duration of this work. I am also very grateful to my associate supervisor, Dr. Jon Hickford, who contributed valuable advice towards the completion of my thesis.

A special thanks to Dr. Jim Morton who unselfishly and unwaveringly furnished support to both my written and experimental work. Thanks also for your friendship, humour, tolerance and seemingly boundless knowledge of both scientific and nonscientific detail.

I would also like to acknowledge Mrs Claire LeCouter who tolerantly proof read all of my work and assisted in its correction. Her excellent technical work has significantly assisted the progress of this laboratory.

A great deal of this work has been affected by the people with whom I spend each day, and I am grateful to them all. Thanks to Andrew Cridge, Mary Clare Craigen, Dr. Lucette Dijkstra, Dr. Geraldine Rogers, Christine Couldrey, Leila Markham, Andrew Escayg, Tania Gourley, Birgit Koch, Mark Lieffering and Ralph Bungard. A very big thanks also to the people who I don't see often enough, Miles Peters, Julian Rayner and Jo Taylor. All you guys have helped more than you probably realise.

Several academic staff have provided valuable assistance at stages of this thesis. Notably, thanks to Drs. Sue Mason, Maurice Barnes, and Chris Malloy for 'on tap' help. I also wish to extend my appreciation to Mrs Jane Brennan who made initiation into practical science easy.

Throughout this endeavour my family has continued to sustain me. I appreciate your tolerance, interest and support. Especially, thanks Mum and Dad, I am very fortunate. Thanks also to my sisters Rosemary, Megan and Susan, and brothers-in-law Ross, Mike and Mark, because without you I wouldn't be able to enjoy my array of nephews and nieces.

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Chapter 1 :

Review of Literature

1.1 Introduction

Meat tenderness is one of the most important characteristics of meat quality (Lawrie 1991), and inconsistency in meat tenderness is a major concern to consumers and producers. Muscle connective tissues (collagen, elastin, reticulin and mucopolysaccharides) are responsible for the 'background toughness' of meat. While the contractile apparatus (actin, myosin, and tropomyosin) is accountable for the more variable 'myofibrillar toughness'. The development of tender meat is incompletely understood, particularly with respect to the biochemical changes that take place. This chapter presents current knowledge of meat tenderisation with a particular focus on proteolysis. Within the conversion of muscle to meat, Geesink (1993) has distinguished two phases shown in Figure 1.1.

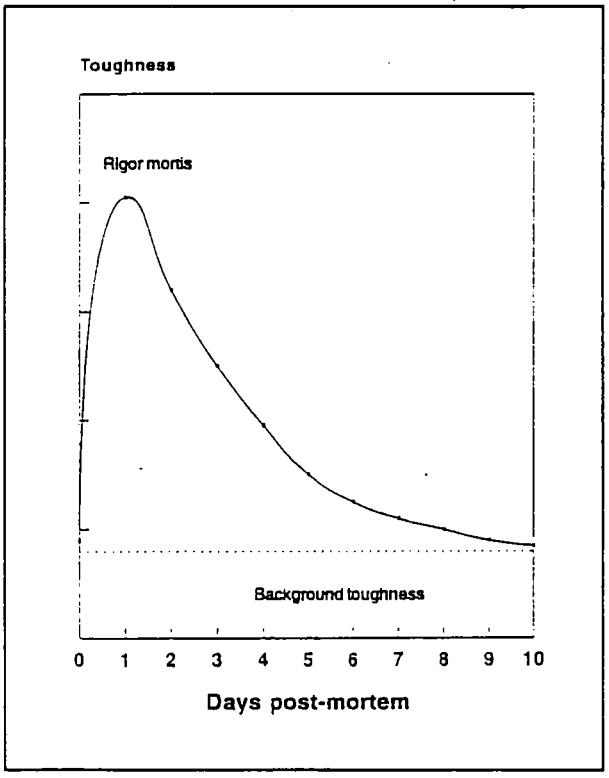

Figure 1.1 A schematic diagram of the effect of ageing on meat tenderness.

The first phase is characterised by the muscle becoming inextensible: this stiffening is referred to as igor mortis or rigor, and is positively correlated with the disappearance of ATP (Lawrie 1991). In he absence of ATP, actin and myosin combine to form rigid chains of actomyosin, this process is

slow at first (delay period), but later becomes more rapid (fast phase). The time required till onset of the fast phase depends upon post-mortem glycolysis, which can resynthesise ATP. Electrical stimulation of whole carcasses is often used to accelerate glycolysis and the onset of rigor mortis. Because blood flow to the muscle has ceased, glycolysis becomes an anaerobic process and produces lactic acid. As a result, tissue pH declines from about 7.2 to 5.5.
The second phase in conversion of meat to muscle is the gradual improvement in tenderness during storage sometimes referred to as conditioning (Etherington 1991). Storing post-mortem muscle (ageing) for up to 3 weeks in a chiller produces noticeable improvements in tenderness and is routinely implemented. However, the method has drawbacks such as the long processing time, storage cost, and the potential for spoilage (Dransfield 1994).

Ultimate tenderness of meat depends on a number of biological and environmental factors such as age, sex, muscle type, stress, protease activity, growth promoters and electrical stimulation. Consequently, the optimum ageing period differs literally from animal to animal and makes prediction of tenderness at any one time difficult. The process of meat tenderisation has been studied intensively over the last 20 years, and an improved although still incomplete understanding of the mechanism of tenderisation has developed.

.2 Muscle conditioning

1.2.1 Skeletal muscle proteins

Skeletal muscles are composed of approximately 75% water, 20% protein and variable amounts of lipid, carbohydrate and other organic compounds (Geesink 1993; Lawrie 1991). The essential structural unit of all muscles is the fibre. These are long, narrow, multineucleated cells which extend through the muscle. They may attain a length of up to 34cm and have diameters of 10-100μm. Within a muscle, fibre diameters vary and usually smaller fibres are peripheral while large are centralised. Each contractile fibre is surrounded by the sarcolemma, and is composed of many myofibrils which contain contractile filaments. Each myofibril is surrounded by the sarcoplasmic reticulum which stores Ca^{2+}.

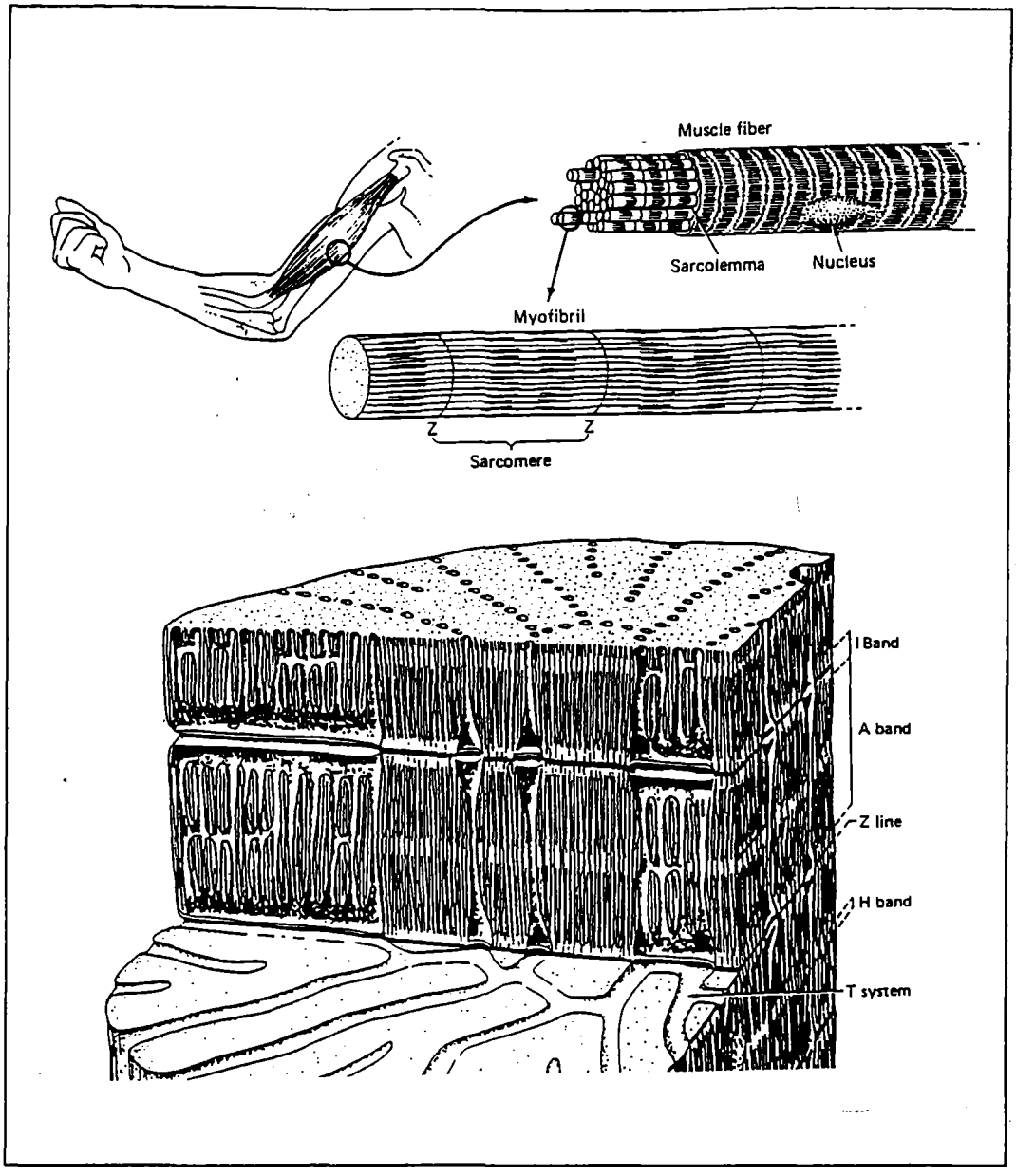

Figure 1.2 A schematic overview of muscle fibre structure (from Hill and Wyse 1989).

he smallest contractile unit is the sarcomere which consists of thick myosin filaments and thin ctin filaments. Approximately 95% of total muscle protein is found in the myofibril, and is lassified as being either sarcoplasmic or myofibrillar. The latter classification is further subdivided ito contractile, regulatory or cytoskeletal proteins (Geesink 1993). Myosin and actin are the main ontractile proteins, while the troponins (C, I and T) and tropomyosin are the regulatory proteins hich control interaction between actin and myosin. Cytoskeletal proteins form a structural etwork within the muscle cell and these include titin and desmin.

1.2.2 Post-mortem changes in muscle

Post-rigor tenderisation during conditioning is not due to breakdown of the actomyosin complex rather, current data suggests that deterioration of key myofibrillar proteins is the principle mechanism behind meat tenderisation (Koohmaraie 1994). Specifically, this involves weakening and degradation of Z-discs at the site of thin (actin) filament attachment (Etherington 1991). Degradation of desmin and titin is seen simultaneously with the increased fragility of muscle fibres during post-mortem storage. A summary of some post-mortem changes in muscles includes:

- Z-disc weakening and degradation, leads to myofibril fragmentation (Suzuki *et al.*, 1982);
- Desmin degradation, leads to myofibril fragmentation (Wheeler *et al.*, 1994);
- Titin degradation, leads to loss of muscle elasticity and connections between myofibrils (Koohmaraie 1994);
- Nebulin degradation, does not effect tenderness;
- Degradation of troponin-T and appearance of polypeptide fragments. Decline in troponin-T does not effect tenderness, however its fragmentation is a good post-mortem proteolysis indicator (Wheeler *et al.*, 1994).

Clearly all these changes require some proteolysis, and today there is little doubt that proteases are responsible for these changes and account for most post-mortem tenderisation (Etherington 1991; Geesink 1993; Uytterhaegen *et al.*, 1994; Dransfield *et al.*, 1992a; Dransfield 1994; Koohmaraie 1994; Whipple *et al.*, 1991).

1.2.3 Proteases involved in post-mortem tenderisation

Koohmaraie (1994) states that for a protease to have a role in post-mortem tenderisation, it must:

- be located in animal muscle cells;
- have access to substrate (ie., myofibrils);
- have the ability to degrade the proteins that are degraded during the tenderisation process.

Three candidates proteolytic mechanisms fulfil these criteria. They are the lysosomal proteases cathepsins), the multicatalytic protease complex and the calpains.

1.2.3.1 The Cathepsins

Cathepsins are distinct from other intracellular proteases in that they designed to function in an acidic milieu, and are localised to the lysosomes (Matthews *et al.*, 1990). It is thought that cathepsins are not likely to play a role in tenderisation for several reasons. The primary substrates of the cathepsins are the contractile proteins (actin and myosin) and breakdown of these proteins is not observed under normal post-mortem storage conditions (Lawrie 1991). Furthermore, cathepsins are sequestered within lysosomes and there is no evidence to suggest that these organelles are ruptured during post-mortem changes (Koohmaraie 1994; Whipple *et al.*, 1991), even after electrical stimulation and storage for 28 days at 4°C (Dransfield 1992a).

1.2.3.2 Multicatalytic protease complex

The multicatalytic protease complex (MCP) is a high molecular weight enzyme (Mr 650-700kDa) composed of multiple units with molecular mass of 22-34kDa (Koohmaraie 1992b; Rivett 1993; Arbona *et al.*, 1993). While MCP is believed to play a major role in extralysosomal proteolysis (Figueiredo-Pereira *et al.*, 1994), its role in muscle protein degradation is uncertain. However, electron microscopic studies would suggest the MCP has no effect on myofibrils (Koohmaraie 1992b), and SDS-PAGE data suggests it only degrades troponin-C and myosin light chain-1 and -2. Thus it is concluded by Koohmaraie (1994) that MCP has little effect on post-mortem tenderisation.

1.2.3.3 The Calpains

There is substantial experimental evidence to indicate a role for the calpains and their inhibitor calpastatin in tenderisation Dransfield 1994; Koohmaraie 1988/1994).

1.3 The Calpain proteolytic system

The calpain proteolytic system (calpain system) is a soluble non-lysosomal protease system responsible for a portion of intracellular protein degradation (for reviews see Rivett 1990; Murachi 1989; Melloni *et al.*, 1991; Koohmaraie 1988). Certain features of this system indicate that it may have a role in intracellular signal transduction. These include the nature of some of its substrates, and the modulation of its activity by calcium. Other possible functions of this system are platelet activation and coagulation (Bassé *et al.*, 1994), apoptosis (Squier *et al.*, 1994), development of Alzheimer's disease (Saito *et al.*, 1993), development of cataracts (David 1993; Azuma *et al.*, 1992) and myoblast fusion during muscle growth (Ebisui *et al.*, 1994).

1.3.1 Components of the Calpain system

Two proteolytic components of the calpain system have been identified (Mellgren *et al.*, 1982). They are alternatively known as calcium activated neutral protease (CANP) I and II, Ca^{2+}-activated protease (CAF, Suzuki *et al.*, 1982), μ-calpain and m-calpain (Murachi 1989), or calpain I and calpain II (EC 3.4.22.17).. This classification is based on contrasts in their sensitivity to calcium (Suzuki *et al.*, 1987). Thus calpain I requires a concentration of Ca^{2+} between 2 and 10μM for maximum *in vitro* activity, whereas calpain II requires a concentration of Ca^{2+} close to 1mM (Salamino *et al.*, 1993). A third element of this system is the specific endogenous inhibitor protein which Murachi (1989) named calpastatin.

The calpains and calpastatin are widely distributed in the animal kingdom, and in addition to mammals and birds they have been identified in fish, crustaceans, and insects (Murachi 1989), plants (Bulaj *et al.*, 1990) and fungi (Huber *et al.*, 1994). The calpain isoforms have been identified in all animal cells examined, with the exception of circulating blood cells (Melloni *et al.*, 1991). Calpastatin is present in all cells containing the two proteases, however, the ratio of these three components is variable between tissues and species (Ouali *et al.*, 1990).

Both calpains (Mr 110kDa) are heterodimeric proteins incorporating a large 80kDa subunit and a smaller 30kDa subunit. Four domains have been identified in the large subunit, and two in the small (see Figure 1.3)

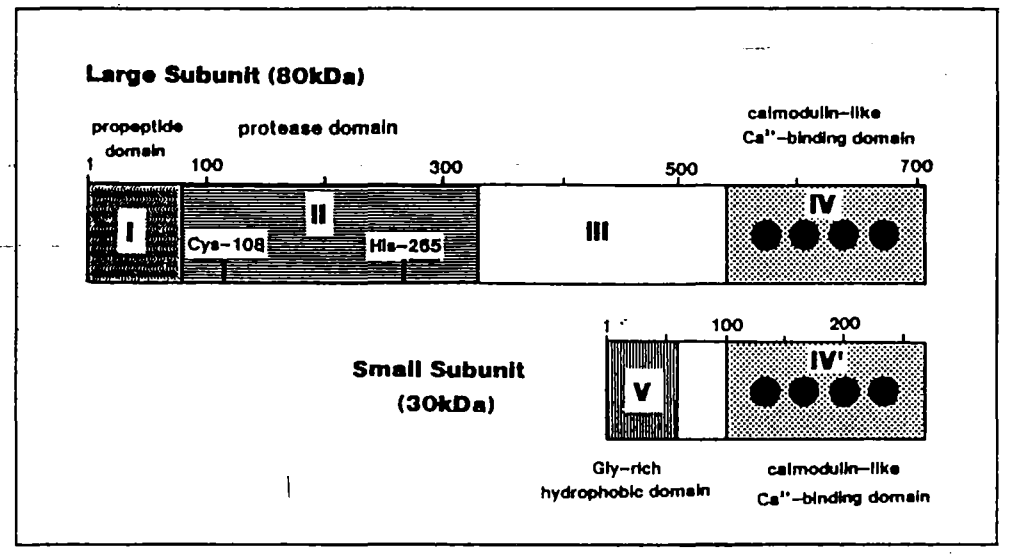

Figure 1.3 Domain structure of calpains I and II comprising a large (80kDa) and a small (30kDa) subunit (Adapted from Suzuki *et al.*, 1992).

Murachi (1989) reports that the 80kDa subunits of calpains I and II are genetically distinct proteins, while the 30kDa subunits are identical. The N-terminal domain I serves two related functions. Firstly, its cleavage possibly promotes conversion of the inactive proenzyme into active enzyme, and secondly its removal liberates the enzyme from its membrane association. The amino acid sequence of domain II resembles other cysteine proteases and is therefore thought to be the enzymes active site. Modification of domain II's structure does not prevent binding of calpain to calpastatin suggesting that interaction does not depend exclusively upon the active site structure (Crawford *et al.*, 1993). Consequently, domain III is thought to be the calpastatin binding region, and facilitates regulation of domain II. Domains IV of the large subunit and IV' of the small, embrace a calmodulin-like amino acid sequence with 4 EF hand structures (Suzuki *et al.*, 1992). The EF hand motif is a reoccuring structure formed by a helix-loop-helix unit which represents the Ca^{2+} binding site in Ca^{2+} binding proteins (Stryer 1988). This suggests a Ca^{2+} binding role for this region. The small subunit has a second domain (domain V) which probably has a regulatory function since its removal increases the sensitivity of the enzyme to Ca^{2+} (Melloni *et al.*, 1991). Interestingly, a minireview recently published by Sorimachi *et al.*, (1994) announced the discovery of several new calpain species. It has been revealed that the large subunit comprises a family of at least six members, which can be categorized as ubiquitous (I- and II- types) or tissue specific (p94/nCL-1 specific for skeletal muscle, NCL-2/-2' specific for stomach) calpains.

Pontremoli *et al.*, (1991) has identified two forms of calpastatin differing in their specificity for calpains I and II. Calpastatin I appears more effective against calpain I, while calpastatin II is effective against calpain II. The two forms can be interconverted by a phosphorylation-dephosphorylation reaction, and *in vivo* this reaction is performed by a CAMP-dependant protein kinase A (Salamino *et al.*, 1994). The rudimentary calpastatin (Mr 105-110kDA) is monomeric and consists of an N-terminal domain (domain L) followed by four homologous domains (domains 1-4). Each domain embraces approximately 140 amino acid residues. By expressing domains 1-4 individually in E.coli plasmids, Murachi (1989) illustrated calpain specific inhibitory activity, in contrast to domain L which displayed none. This suggests it is these homologous regions that confer inhibitory potential to the protein. Each domain (1-4) can be divided into 4 subdomains (eg., 1A, 1B, 1C and 1D) composed of 27-33 residues. An experiment performed by Maki *et al.*, (1989) established that a chemically synthesised 27 residue oligopeptide of domain 1B possessed potent inhibitory potential. This endorses the report that calpastatin can be fragmented by calpain into peptides which display almost complete inhibitory potential (Melloni *et al.*, 1991). In theory, therefore, one calpastatin should be capable of inhibiting four calpain molecules. However, this stoichiometric relationship is not typical in either recreated systems or in intact cells. Ca^{2+} is required for the inhibition of calpain by calpastatin (Crawford *et al.*, 1993), and the mode of

inhibition is competitive and fully reversible, leading to a fully active enzymes upon dissociation.

1.3.2 Activation of the Calpain protease system

There is general agreement that the mechanism for activation of calpain protease requires the conversion of inactive proenzyme to active enzyme via autoproteolysis (McClelland *et al.*, 1989; Melloni *et al.*, 1984). Specifically, this involves the cleavage of domain I (Suzuki *et al.*, 1992), and possibly domain V (Melloni *et al.*, 1991) from both, or either, subunits. This conversion is dependant upon relatively high Ca^{2+} concentrations (ie., 5mM for calpain II and 1mM for calpain I (Nishimura *et al.*, 1991)) indicating a prerequisite role for this ion (McClelland *et al.*, 1989). However, the availability of substrate (eg., casein) lowers the Ca^{2+} demands to approximately 5μM (Melloni *et al.*, 1984/1991). The initial cleavage generates an 18kDa C-terminal fragment (Crawford *et al.*, 1993) which appears to contain the entire domain VI from the 30kDa subunit (Nishimura *et al.*, 1991). Simultaneously, a 78kDa subunit is detectable. Over longer periods of incubation (ie., up to 90min at 25°C) 55, 34, 27 and 21 kDa fragments appear, the 34 and 21kDa remnants are the major products of autolysis. The former contains all of domain II, the catalytic domain, and some of domain I. The latter embraces domain IV, the Ca^{2+} binding region, and about 50 amino acids from domain III of the large subunit (Nashimura *et al.*, 1991). As a consequence of this initial autoproteolysis, the enzyme requires lower physiological concentrations of Ca^{2+} for activation (Edmunds *et al.*, 1991), and has the potential to operate *in vitro.* The initial self activation requires a concentration of Ca^{2+} approximately two orders of magnitude higher than that normally present in cells (Salamino *et al.*, 1993). Therefore, it is reasonable to assume that a mechanism exists that is capable of increasing the affinity of the protease to Ca^{2+} *in vivo.*

Melloni *et al.*, (1991) has proposed an activation model (Figure 1.4) where external stimuli lead to transient rises in intracellular Ca^{2+}. Under these slightly elevated Ca^{2+} concentrations, calpain is translocated to the inner surface of the plasma membrane where interaction with the lipid bilayer encourages a conformation change (Pontremoli *et al.*, 1985; Gopalakrishna *et al.*, 1985). This promotes autoproteolysis at normal physiological Ca^{2+} levels. Cleavage of the anchoring domain releases the enzyme into the cytosol. Under the translocation-activation theory cytoskeletal proteins, transmembrane or intrinsic proteins, and proteins translocated to either the plasma membrane or the cytoskeletal array all become potential calpain substrates. A second, and possibly related mechanism suggests that the inactive proenzyme associates with a low molecular weight (40kDa) activator protein at the plasma membrane (Salamino *et al.*, 1993). This association converts the proenzyme to the low Ca^{2+} requiring isoform which then dissociates and becomes fully active.

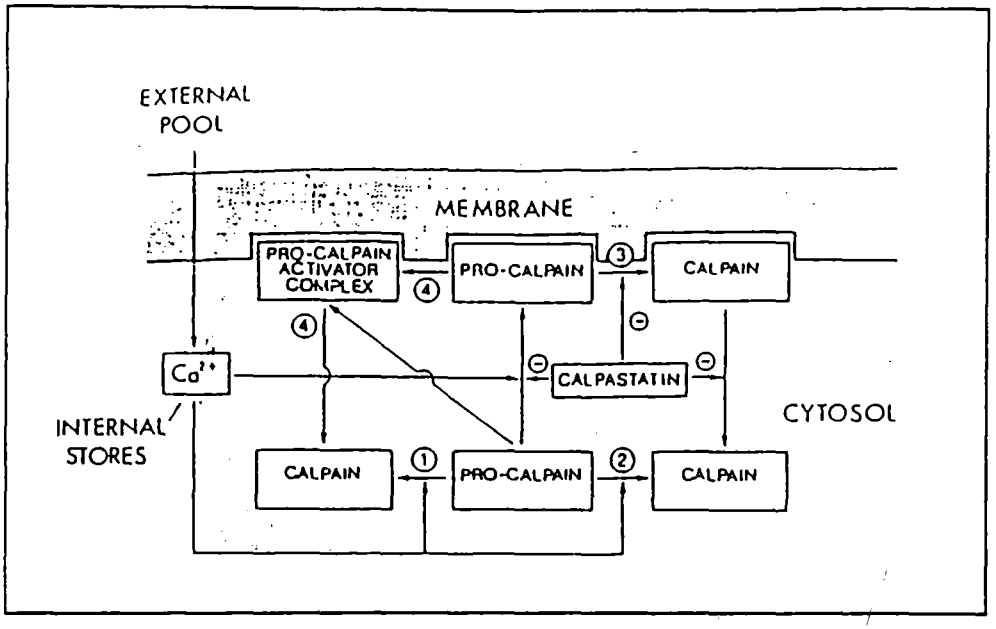

Figure 1.4 Model for *in vivo* calpain activation (from Melloni *et al.*, 1991). Pro-calpain refers to the inactive proenzyme form. Numbers (circled) indicate activation by 1) abnormal increase in Ca^{2+}; 2)low Ca^{2+} and a digestible substrate; 3) interaction with plasma membrane; 4) interaction with activator protein. The Θ symbol denotes points at which calpastatin may inhibit the process.

Understanding the calpain/calpastatin system *in vivo* is complicated by the fact that both components (protease and inhibitor) are found in the same cellular compartments. Figure 1.4 displays several positions in the activation model where calpastatin may inhibit calpain. For example, calpastatin may prevent translocation of procalpain to the plasma membrane, or the conversion of procalpain to calpain via the association with membrane phospholipids. It is not clear how, or if, calpains avoid inhibition by calpastatin, but there are a number of possibilities. It could be that a large quantity of calpain relative to calpastatin enables some free, unbound calpain to operate. Imbalances of this nature have been detected in the human pituitary (Kitahara *et al.*, 1986). Localisation at the plasma membrane may sequester calpain away from calpastatin (Gopalakrishna *et al.*, 1986), and formation of the enzyme-inhibitor complex may demand higher Ca^{2+} concentrations than those required for enzyme activation alone. It is, however, conceivable that under normal *in vivo* conditions calpain is completely inhibited by calpastatin. Should intracellular Ca^{2+} concentration rise to an abnormal level then the calpains are activated excessively and override calpastatin inhibition. As a consequence autoproteolysis and deregulated protein catabolism occurs within the cytosol. Calpain II has the potential to degrade calpastatin during the unregulated proteolysis (Melloni *et al.*, 1991).

This model of activation is not universally agreed upon. Goll *et al.*, (1992) has suggested that calpains do not bind to phospholipids at the plasma membrane, but rather the associate with

specific proteins such as phospholipase C-ß (Banno *et al.*, 1994). Furthermore, this association does not reduce the Ca^{2+} requirement for activation. Goll *et al.*, (1992) suggests that regulation of calpain activity is achieved by Ca^{2+} binding to certain sites on the calpain molecule which elicits a variable response (eg., activation, calpastatin binding). How this system is regulated is unknown at this stage. Additionally, an article by Crawford *et al.*, (1993) however, has stated that for calpain II to display activity, autoproteolysis is not necessary and calpain II does not exist as a proenzyme. Apparently, some questions still exist regarding calpain activation.

1.3.3 Involvement of calpains in post-mortem tenderisation.

There is strong evidence to suggest calpains are involved in post-mortem tenderisation (Dransfield 1994). Calcium is known to have catabolic effects on protein turnover (Lewis *et al.*, 1982) and tenderisation can be accelerated and improved by addition of Ca^{2+} ions (Wheeler *et al.*, 1991b; Whipple *et al.*, 1992). Conversely, myofibril fragmentation can be inhibited by EDTA, zinc and leupeptin (Uytterhaegen *et al.*, 1994; Whipple *et al.*, 1991; Koohmaraie 1990a/1992a). Zinc and leupeptin both inhibit cysteine proteases. Additionally, *in vivo* myofibrillar changes occur in parallel with the activity of calpain I during the 24 hour period immediately post-mortem (Koohmaraie *et al.*, 1987), and significant correlations have been reported between calpain activity and myofibril fragmentation (Shackelford *et al.*, 1991). Finally, Forsberg *et al.*, (1989) infused rabbits with a ß-adrenergic agonist (cimaterol) and observed a corresponding reduction in myofibrillar protein degradation and increase in calpain activity. This has been substantiated by Kim *et al.*, (1993) and Wheeler *et al.*, (1992). Thus there is substantial evidence to indicate the calpain system has an important role in determining meat tenderness.

1.3.3.1 Factors influencing the activity of the calpain system

The amount and activity of calpains varies according to important biochemical regulators such as changes in Ca^{2+} concentrations, binding of calpain to calpastatin, inactivation of free calpain, and the proteolysis of calpastatin (Dransfield 1993). However, other factors such as storage conditions, rigor development and animal production may dictate these biochemical influences (Dransfield 1994). With respect to animal production, little research has been performed to establish whether growth manipulation has an effect on the tenderness of meat or the calpain proteolytic system.

1.4 Biotechnology

Currently there is a trend towards producing leaner meat in response to consumer demands (Campion *et al.*, 1991). Traditionally, the selection and cross breeding of animals has been used to manipulate growth, but recent advances in genetic engineering and molecular biology have provided new innovative technologies. de Vries *et al.*, (1991) has classified these into four biotechnological approaches. They are;

- **use of DNA markers**: the search for genetic polymorphism which leads to identification of genes linked to desirable traits such as superior growth and development. This permits the selective breeding of animals based on genotype rather than phenotype;
- **improved reproduction techniques**: used to increase reproduction rates, number of offspring per animal, and in concert with the use of DNA markers the best embryos from a choice of several;
- **production of transgenic animals**: transgenic animals result from the incorporation of foreign genes into early embryos. This produces genetically modified offspring;
- **application of biotechnological products**: administration of biotechnological products (eg., hormones and antibodies) to modify animal hormonal status and promote growth.

Some important distinctions can be made between these approaches based upon their various advantages and disadvantages. Genetic modifications, such as transgenic engineering and selective breeding based upon DNA markers, are intended to make a positive contribution to the gene pool and are long lasting. Whereas administration of biotechnological products such as hormones and antibodies produce a more immediate and transient effect, are usually cheaper, and the products do not affect the genotype.

One problem affecting all of these approaches is that there is an incomplete understanding of the biology of growth. Specifically, within an animal there are several complex interrelated systems responsible for growth and modification of one may not necessarily produce the desired phenotypic change. This lack of understanding extends from the control of genes to the action of hormones. Furthermore, there are several technical difficulties associated with the reproductive biology of production animals such as recovery of eggs for microinjection, long gestation times and relatively small litter sizes of commercially important livestock such as cattle and sheep (Smith *et al.*, 1991). Whilst the creation of transgenic animals is a technology that is currently unreliable, expensive and has a low efficiency for producing first generation transgenic animals (de Vries *et al.*, 1991). Additionally, it is probably naive to assume that the carefully considered insertion, deletion or

modification of a gene will not impact on other metabolic processes. Any unintended effects must be identified in case they are harmful to the animal or consumer, and depending on the nature of the effect this confirmation can be difficult, expensive and time consuming. Finally, despite the potential of biotechnology there are profound moral, ethical and safety questions of public concern that need to be taken into account (Grandin 1991). Consequently, the application of biotechnology techniques must be considered carefully, and acceptability schemes employed (Lupien 1991).

Immunomodification is a technique included under the 'administration of biotechnological products' classification. It is characterised by alteration of the target animals immune system, which leads to further metabolic changes. Usually, immunomodification seeks to change an animals hormonal status with the intent of promoting growth.

1.4.1 Immunomodification

Pell *et al.*, (1991) summarises the manner in which immunomodification may be employed. Firstly, animals may be vaccinated against proteins such as SRIF (which inhibits growth hormone production) in the same way humans are vaccinated against viral and bacterial diseases using an immunogenic fragment causing **neutralisation of activity**. Vaccination depends upon the endogenous production of polyclonal antibodies which can neutralise endogenous, or 'self', hormone activity.

Next, monoclonal antibodies (mAbs) can be produced from mice which recognise specific proteins or peptides associated with hormone activity, injection of these into livestock animals can actually enhance the activity of these proteins. Similarly, the injection of peptides involved in growth (eg., the active site of growth hormone) leads to the production of endogenous polyclonal antibodies which can augment or **potentiate** the activity of the peptide in a similar manner to Mabs.

Finally, antibodies can be used to mimic hormones or peptides, and these encourage anti-antibodies (or anti-idiotypes) to perform as the original hormone. An anti-idiotypic response may arise following administration of an enhancing peptide (eg., the active site of growth hormone) to an animal. Because the enhancing peptide is a truncated form of the whole protein it is not recognised as 'self' and induces the generation of antibody 1 (Ab1) which specifically recognises this active site. This is occurrence is analogous to a glove around a hand (Holder *et al.*, 1991). Ab1 now displays a region that is the negative of the enhancing peptide. Ab2 is generated against Ab1 molecule in a similar glove and hand fashion, this returns the negative to a positive, and Ab2 may then bind to the GH receptor and generate a GH-like response in the cell. This process is referred to as an **anti-idiotype response** (Pell *et al.*, 1991; Holder *et al.*, 1991).

1.4.2 The Growth Hormone Axis

One facet of the endocrine system that is often targeted for manipulation by immunomodulation is the Growth Hormone Axis. This axis has direct and indirect actions on growth regulation and regulates the distribution of fat and protein between body tissues.

Growth hormone (GH), also known as somatotropin, is synthesised by the anterior pituitary. Direct effects are mediated by growth hormone binding to the growth hormone receptors on the surface of target cells. Indirect effects are mediated by stimulating the synthesis of Insulin like Growth Factor's (IGF's, often known as somatomedins) which bind to IGF receptors. The effects of GH vary according to different tissues (Aston *et al.*, 1991). This diversity of action is attributable to the differences in the structure of the GH receptor and by post-translational modifications of the receptor molecule.

The synthesis of growth hormone in the anterior pituitary is under the influence of positive and negative regulators from the hypothalamus (Phillips 1987). The regulators have been characterised and are called somatotropin release inhibiting factor (SRIF, a negative effector) and growth hormone releasing hormone (GHRH, a positive effector). The control of GH release is also under the influence of negative feedback loops which inhibit GH production at the pituitary and encourage SRIF synthesis. The IGF's produced by GH stimulation inhibit GHRH and augment the activity of SRIF (Rudd 1991) and GH by down regulating GH production.

The effects of GH were first identified 50 years ago by Evans and Simpson (1931). In their trials, crude GH was repeatedly administered to rats and an increase in body weight ensued. Upon closer examination it was discovered that protein accretion had increased and fat deposition was reduced. Since this time, a list of GH effects have been reported. Notably, administration of GH to sheep, pigs and cattle increases growth rates (Pell *et al.*, 1991) and reduces the lipid content of whole carcasses (Dawson *et al.*, 1991; Wood *et al.*, 1991). An increase in total muscle has also been observed (Pommier *et al.*, 1990; Pell *et al.*, 1991) in addition to diabetogenic and lactogenic effects.

The exact mechanism by which GH effects growth remains unclear. As already noted, GH may operate directly upon tissues or by encouraging the production of secondary growth promoters eg., somatomedins. Most of the GH effects on muscle are probably mediated by IGF-1 which increases in response to GH administration (Wallis *et al.*, 1987). No direct effects of GH on muscle cells have been demonstrated but IGF-1 has been shown to promote amino acid uptake, muscle cell proliferation and glucose uptake. (Dawson *et al.*, 1991)

1.4.3 GH and immunomodification

Administration of exogenous growth hormone has been shown to significantly increase animal growth (Pell *et al.*, 1991). However, consumer demands for wholesome products and the industry concern over public safety have resulted in the technique not being universally accepted, and it is unlikely to be used commercially (Holder *et al.*, 1991).

In contrast, over the last 10-15 years the immunological manipulation of factors associated with the GH axis (GH, SRIF and GHRH) has attracted considerable attention, and mAb enhancement of GH action has been extensively studied (Holder *et al.*, 1991). Typically, a decrease in fat deposition and an increase in whole body protein occurs. Additionally however, an increase in IGF-1 levels is observed with GH changes, suggesting that the body composition changes are taking place via normal growth pathways. Included in the list of effects resulting from immunomodification to augment GH are :-

- lactogenic effects (Holder *et al.*, 1988; Pell *et al.*, 1989);
- somatogenic effects (Holder *et al.*, 1988);
- diabetagenic effects (Pell *et al.*, 1989);
- increases in serum GH (Gardner *et al.*, 1990; Dawson *et al.*, 1991);
- increases in GH activity (Aston *et al.*, 1986; Aston *et al.*, 1991);
- increases in circulating IGF-1 levels (Dawson *et al.*, 1991; Gardner *et al.*, 1990; Wallis *et al.*, 1987);
- augmented growth rate and whole body protein content (Aston *et al.*, 1991);
- a rise in average body weight (Gardner *et al.*, 1990; Dawson *et al.*, 1991; Holder *et al.*, 1988; Wallis *et al.*, 1987);
- changes in body composition and increases in feed consumption (Holder *et al.*, 1988);
- reductions in body fat content (Holder *et al.*, 1988).

Additionally, however, immunomodification has been employed to modify animal growth via targeting the regulators (SRIF and GHRH) and effectors of GH (IGF's).
Using GHRH conjugated with human serum albumin, it is possible to induce an immune response against this hormone. Immunoneutralization in this way leads to a number of metabolic changes in cattle. Firstly, the normal episodic release of GH from the pituitary is disrupted, and the basal concentration and frequency of GH release was lower than that of the control animals. Additionally, IGF-I concentrations were reduced (Moore *et al.*, 1992). These changes were reflected in a lower growth rate and final animal size.

Reducing the activity of GH inhibitors such as somatostatin (SRIF) has been demonstrated by Spencer (*et al.*, 1983) who found that administration of a somatostatin/human serum globulin complex to lambs induced an immune response against somatostatin. This coincided with a significantly greater weight gain and mature height in treated animals when compared to control animals ($P<0.001$). Increases in GH, insulin and IGF's were also noted. Weight gain was proportional in all tissues ie., there was no changes in body composition. In a similar study, immunisation against somatostatin was performed by obtaining commercial somatostatin and conjugating this with bovine thyroglobulin (Bass *et al.*, 1987). This adjuvant was injected into sheep and elicited an immune response. The immunisation caused a small increase in growth rate but had no effect on body composition. No effect was seen on the plasma concentrations of GH, IGF-II, free fatty acids or plasma glucose but IGF-I concentrations were raised. In trials performed by Pell (*et al.*, 1991), immunoneutralisation against somatostatin induced a 76% improvement in whole body growth compared to globulin immunised controls. Again there was no alteration in the proportion of bone, muscle and fat. Experiments with protein conjugated somatostatin in pigs has shown that GH levels were augmented compared to control animals (Dubreuil *et al.*, 1989). However, Holder (*et al.*, 1991) noted that immunisation against somatostatin has generated conflicting results. Some papers claim an increase in GH occurs, whilst others report a drop in growth rate. It would appear that variables such as sex, age, breed etc have compounding effects on somatostatin immunisation. Indeed subsequent experiments by Pell (*et al.*, 1991) have not substantiated the preliminary trials thus immunoneutralisation against somatostatin has remained controversial.

Passive immunisation to neutralise circulating IGF-I was investigated to determine how important its mode of secretion is in maintaining animal growth (Kerr *et al.*, 1990). Immunisation against IGF-I had no net effect on body metabolism. However an increased circulating IGF-I level was observed. The lack of an immunisation effects suggests that local IGF-I synthesis is sufficient to maintain growth or that local IGF-I production is modified to maintain homeostatic levels.

1.4.3.1 Mechanism of antibody potentiation

There are several theories based on the above observations that attempt to explain how a GH-antibody association leads to potentiation of GH activity.

Firstly, it has been suggested that Mabs increase the circulating half life of GH by limiting the rate of normal hormone degradation (Pell *et al.*, 1991; Kerr *et al.*, 1990). However, currently there is no correlation between this observation and the ability of Mabs to potentiate or promote growth (Wallis *et al.*, 1987).

Antibodies may induce a conformational change in the structure of GH leading to an elevation in activity of GH. However, this is unlikely because Mabs specific for different regions (epitopes) of the GH molecule amplify the effects of GH similarly (Holder *et al.*, 1991), and for this to happen all the Mabs would have to induce favourable conformational changes in the molecule.
The GH-antibody complex may prolong the receptor association (Holder *et al.*, 1988) by limiting the normal process of receptor processing and degradation. This would reduce the receptor turnover and increase the intracellular signal (Holder *et al.*, 1991). Alternatively, different receptors exist for the GH molecule and Mabs raised against GH may restrict Mab-GH binding to only some of the receptors. This may lead to some receptors being unoccupied and receptive to other GH molecules (Holder *et al.*, 1988).
It is also possible that the Mab-GH complex binds to receptors that are more biologically active and as a consequence a greater growth response occurs (Wallis *et al.*, 1987). This theory is called the "restriction hypothesis" (Aston *et al.*, 1991). It is supported by evidence presented by Aston (*et al.*, 1986), EB1 and EB2 are Mabs specific for different GH epitopes. Both have in vivo enhancing effects on GH activity but when administered *in vitro* to cell lines such as RLM and IM9 lymphocytes, the effects on GH are inhibitory. This suggests that the Mabs bind to separate GH receptors with different growth promoting potential. It is suggested that the differences between in vivo and in vitro studies may be attributed to Mabs restricting the binding of GH to distinct receptors.

While the mechanism(s) by which antibody mediated growth promotion remains unclear, several hypotheses have been presented. All the mechanisms involve aspects of basic hormone action since responses to antibody treatment are analogous across various hormone experiments (Holder *et al.*, 1988).

1.4.3.2 Immunomodulation and animal production

Public concern over the fat content and price of meat has encouraged the development of technologies that are capable of increasing muscle accretion (Dawson *et al.*, 1991). In reality, the exogenous administration of GH or any other hormone will probably never be commercially practised due to consumer resistance and safety concerns. This is despite the fact that compounds such as bGH and pGH have no effect on primates and possess relatively short half lives (Aston *et al.*, 1991). A more acceptable technique is the use of immunisation to produce endogenous factors, such as hormone specific antibodies, which will modify the endocrine system. There are two important favourable characteristics of immunomodulation in commercial livestock. Firstly a limited number of small injections can induce a relatively large immune response. In contrast, the

administration of synthetic GH requires regular injections producing an acute response (Pell *et al.*, 1991). Secondly, if the growth effector (eg., GH active site) is successful in eliciting an immune response then it will stimulate the production of specific polyclonal antibodies. These may then bind to, and potentiate, the action of whole endogenous GH through one of the mechanisms described above (Holder *et al.*, 1991). Mab complexes cannot be considered endogenous since the Mab probably originates from mice, consequently polyclonal potentiation may be perceived as being more "natural". Public education campaigns should be encouraged to illustrate the advantages of this technique if this technology is to be pursued further.

1.5 Separation of the Calpains using chromatography

Chromatography techniques are used to separate, purify and identify compounds from a mixture of elements. Generally chromatography protocols involve two phases, a stationary phase and a mobile phase which moves over, through or around the stationary phase (Wilson and Goulding., 1986; Scopes., 1987). The mobile phase represents compound mixture (eg., proteins), while the former phase describes some sort of absorptive material to which the compounds may differentially associate. Many stationary phase materials are available and usually one is selected on the basis of its potential to bind to a particular compound of interest and thus remove it from the mixture. Many common stationary phase materials operate on the principles of ionic exchange and hydrophobic interaction.

1.5.1 Ion-exchange chromatography

The underlying principle of this technique is the attraction of oppositely charged molecules for one another. Most proteins contain ionisable groups which furnish the molecule a net positive or negative charge. The matrices employed in ion-exchange chromatography present charged groupings to which proteins may attach (Wilson and Goulding 1986). Diethylaminoethyl Sephacel (DEAE Sephacel) the ion-exchange matrix used in these experiments. Its molecular structure includes a polysaccharide chain (cellulose) to which DEAE groups have been attached. Importantly, DEAE contains a nitrogen group which confers a positive charge to the matrix; this will associate with anions in the mobile phase. The modified molecule is correctly known as a weak anion exchanger (Clark and Switzer 1977) because the net charge on the DEAE molecule is positive and thus its exchangeable counter ions are negative. Elution of proteins ionically bound to the matrix material can be achieved by changing the ionic strength or pH of the buffer washing through the column.

DEAE sephacel chromatography is a commonly employed chromatographic approach used for the separation of the calpain system components, and a number of studies have been performed to demonstrate its abilities (Koohmaraie 1990b; Wheeler *et al.*, 1991a). Generally, tissue samples are prepared in buffers which contain agents to limit autolysis (eg., EDTA, EGTA, leupeptin). Care is taken to minimise the ionic strength of the buffer so that non-protein charged groups (eg., salts) will not limit the desired protein-DEAE associations. The adjustment of ionic strength of the buffer fulfils another useful purpose in that it standardises the initial ionic concentration at which binding takes place. With respect to the extraction of the calpains this is usually less than 25mM NaCl (Wheeler *et al.*, 1991a). This then permits accurate reproductions of the ionic changes required to elute off the desired proteins. Furthermore, a well controlled ionic strength can be used to prevent weakly binding material from associating with the DEAE groups even before a elution protocol is applied. This initial fractionation of the sample can greatly reduce the A_{278} absorbing material and make the elution profile 'cleaner'.

1.5.2 Hydrophobic chromatography

Phenyl Sepharose operates on the basis of hydrophobic interactions (or salt promoted adsorption (Scopes 1987)) as opposed to ionic interactions. Proteins are generally soluble in water or dilute salt solutions but most contain aliphatic or aromatic amino acids that are not water soluble. The proteins solubility is achieved by the protein folding around clusters of hydrophobic residues, thus isolating them from the proteins environment. This structure is maintained by weak van der Waals associations, and the net energy gained from such an arrangement contributes to the overall folding of the protein (Clark and Switzer 1977). These internal hydrophobic groups will only be exposed if the molecule is disrupted in some way, otherwise the interaction with chromatography matrices must rely on the surface hydrophobic regions. Scopes (1987) notes that although most hydrophobic groups tend to be held inside the molecule, substantial numbers reside on the protein surface and are exposed to the solvent. These are variable in number and vary in strength from protein to protein. The functional group of phenyl sepharose is a benzene ring, linked by glycerol ether to the backbone molecule, agarose. Agarose is found naturally in agar. However agar contains many charged and neutral groupings. These are removed in the generation of agarose to leave a polysaccharide molecule possessing few charged groupings. Elution is achieved by disrupting the structure of water and thus discouraging hydrophobic interactions or by removing agents (such as salt) that are augmenting the hydrophobic associations. In the former case, elution agents include ethylene glycol or ethanol (Wilson and Goulding 1986) or other chaotrophic agents.

Three different approaches, have been used to separate calcium dependant proteases and calpastatin

from muscle. The differences between the methods are based on the chemical attributes of the calpain molecules. Specifically their propensity to unfold and expose hydrophobic domains in the presence of Ca^{2+}. Gopalakrishna and Barsky (1986) noted that following calpain I and II binding to Ca^{2+}, they both expose a strongly hydrophobic core domain. This behaviour is not unique and has also been witnessed in other calcium binding molecules possessing the EF hand motif eg., calmodulin and troponin C.

This Ca^{2+} induced behaviour is exploited in **Ca^{2+} dependant hydrophobic chromatography** where the calpain containing extract is filtered through a column of phenyl sepharose beads. In the presence of $CaCl_2$ the conformation of the molecule is altered to expose its internal hydrophobic regions. These then associate with the hydrophobic domains on the phenyl sepharose beads, and separation from non-hydrophobic proteins is achieved. This technique demands the inclusion of a protease inhibitor (eg., leupeptin) to prevent the calpains autolysing in the presence of Ca^{2+} (Ou *et al.*, 1991; Ilian and Forsberg 1992; Gopalakrishna and Barskey 1985). Furthermore, the inhibitor must be washed from the column before elution of the calpain fractions. Extraction from the column is achieved by introducing a Ca^{2+} chelating agent, such as EGTA, which complexes out Ca^{2+} and reverses the Ca^{2+} induced calpain/bead association.

An alternative separation technique makes use of much weaker **non-Ca^{2+} dependant hydrophobic chromatography** principle. This technique takes advantage of surface hydrophobic residues which naturally bind to phenyl sepharose, but with considerably less affinity than those groups exposed by the addition of calcium. Hodgkinson and Lowry (1981) have noted that hydrophobic interactions generally increase with increasing ionic strength, and the addition of NaCl to the calpain extract promotes salting out of the calpain molecules onto the sepharose beads (Gopalakrishna and Barskey 1985/1986). Elution is achieved by successive washes which lower the NaCl concentration of the fluid medium.

Difficulties exist in both the Ca^{2+} dependant and Ca^{2+} independent chromatography separation of calpains and calpastatin. In the case of Ca^{2+} dependant techniques, the use of EGTA to elute off the calpains produces only a single peak of protease (Gopalakrishna and Barskey 1985) and separation of the two calpain isoforms requires further separation eg., DEAE Sephacel. Additionally, calpains form complexes with calpastatin and other endogenous substrates in the presence of Ca^{2+}. These associations can limit the binding of calpain to the phenyl sepharose beads. The Ca^{2+} independent technique employs the use of relatively strong ionic mediums (eg., 0.5M), high NaCl concentrations which can lead to the inactivation of calpain II, even in the presence of protease inhibitors (Gopalakrishna and Barsky 1985; Kendall *et al.*, 1993). These

complications necessitated the development of a third technique termed the **routine isolation procedure** (Gopalakrishna and Barsky 1985). This technique utilises aspects from both of the previous procedures. Incubation of calpain proteins with Ca^{2+} and a strong ambient NaCl concentration promotes initial binding to the matrix. Elution of protein off the column is achieved by washing with decreasing NaCl concentrations in the presence of EGTA ie., Calcium independent elution.

1.6 Summary

The calpains are part of an endogenous intracellular proteolytic system found in all cells. The activity of these enzymes has been associated with structural changes of the myofibril during post-mortem muscle conditioning, and currently there is a widely held view that the calpains are responsible for post-mortem tenderisation of meat. The use of immunomodification to augment the growth of livestock is a promising technique with several favourable characteristics. However, no researchers have yet investigated the effect of this manipulation upon tenderness and calpain activity. This thesis intends to assess this question by using chromatographic techniques to isolate the components of the calpain system, and comparing proteolytic activities with tenderness.

Chapter 2
Aims of Present Study.

As explained in chapter one, tenderisation is a complex multifactorial process, and several environmental and animal based factors influence the conditioning of muscle into meat. The calpain proteases, and their endogenous inhibitor calpastatin, are important biochemical factors whose activities profoundly effect meat tenderness (Koohmaraie 1994; Dransfield 1994) and hence quality. At this stage our understanding of the behaviour of these proteases is incomplete, particularly with respect to their normal physiological roles and the factors that regulate them. A complete appreciation of the calpain system, once developed, would enable researchers to advise producers and processors of meat how to maximise tenderness and minimise variations in tenderness of meat products. A solid foundation of knowledge will also enable researchers to plan future experiments more effectively.

The aim of this study is to better understand the behaviour of the calpain enzyme system in ovine skeletal muscle. In particular, it will try to optimise methods for their extraction and quantification, investigate their *in vivo* behaviour with ageing, and their associations with parameters such as body composition. Five experimental chapters are included in this thesis. A brief description of the aims of each chapter follows:

Chapter 3 - Comparison of methods for the extraction and measurement of the calpain proteases and their inhibitor calpastatin.

This chapter evaluates several existing techniques that are used to extract and measure calpains and calpastatin. A comprehensive survey of the literature revealed that four distinct techniques are employed by researchers. Two of these involve hydrophobic chromatography and two utilise ion exchange chromatography. These techniques have been reproduced as described in the papers and specific elements of the protocols evaluated. Factors taken into consideration are the ability of the procedures to separate the proteases from muscle, their technical ease, reproducibility, speed of separation and the yield of proteases and inhibitor.

Chapter 4 - Development of an extraction and measurement technique suitable for the detection of calpains and calpastatin.

The characteristics of a "good" experimental technique are that it is reliable and reproducible. However, consideration of the time involved and convenience of the technique should also be taken into account. The results from chapter 3 suggest a procedure that fulfils these requirements. This chapter relates a standardised analytical protocol for the extraction and measurement of the calpain system components to be used in later experiments described in the thesis.

Chapter 5 - Characterisation of calpain activities over time in post-mortem ovine muscle.

In accordance with the development of a better understanding of the calpain enzyme system, this experiment will investigate changes in the calpain/calpastatin system in tenderisation during storage of post-mortem meat.

Chapter 6 - Differences in the activity of calpains and calpastatin in two lines of sheep differing in their glucose tolerance.

Levels of circulating glucose are known to have an effect on growth hormone concentrations via mediation of insulin levels. This chapter examined whether differences in glucose tolerance, insulin levels, and body composition were reflected in differences in the calpain protease system. Two lines of genetically selected sheep, one displaying high glucose tolerance and one low, were used in this work. The associations between body composition and components of the calpain system were measured and compared.

Chapter 7 - The effect of immunisation against growth hormone on components of the calpain protease system in sheep.

Several studies have demonstrated the potential of monoclonal antibodies specific for growth hormone to modify growth and body composition in a variety of animals including sheep. This chapter establishes whether observed differences in body composition, resulting from antibody mediated growth hormone modification are associated with changes in the calpain protease system. The use of antibodies to

increase animal growth has received considerable support and has important ramifications for the meat producing industry. No work has examined whether modifications of growth by growth hormone antibodies has an effect on the proteolytic enzymes or meat tenderness.

Chapter 3:

Comparison of Methods for the Extraction and Measurement of the Calpain Proteases and their Inhibitor Calpastatin.

3.1 Introduction.

There is now considerable evidence to support that the calpain proteases and their inhibitor calpastatin are involved in the conditioning of meat and may control the ultimate tenderness of meat (Koohmaraie 1994; Dransfield 1994). Studies have variously implicated calpain/calpastatin activities, abundance, ratios and structures as being responsible for the differences in meat tenderness observed between individuals and species. Whatever the exact nature of the calpain system and tenderness, all research in this field has required reliable, reproducible and accurate techniques for the extraction and quantification of the components of the calpain system.

A variety of experimental approaches have been used to elucidate the structure of the proteins and observe changes in the conformation of the calpains resulting from autolysis. Other research has been concerned with quantification of components associated with tenderness predictors such as treatments (eg exogenous growth hormone), time, or species differences. In the former experiments purification of the native protein is demanded, and obtaining a complete yield of the protein from a muscle source is usually not vital. In the latter types of experiments, however, a relatively crude protein preparation is satisfactory and a complete quantification of the proteins is required. Obviously, the nature of the experiment and the information desired from the experiment dictates the experimental techniques used.

In the experiments contained in this thesis, a total yield of protein from a sample is required but the absolute purity of the isolate is not crucial. This is because these experiments are concerned with revealing changes in protein activity associated with treatments and the effects of parameters such as time, processing, glucose tolerance and circulating growth hormone status of the animals. Thus the survey of techniques is limited to those whose ultimate objectives are similar.

In this chapter five methods of calpain extraction were compared in order to determine which

would be the most useful in future experiments. Four original methods were examined, each reproduced as described in the literature. A fifth method, which was modification of one of the original methods, was also examined. The five methods were judged primarily upon their reliability, reproducibility, and accuracy. Other factors considered were the time required for analysis, cost, and the labour involved.

All approaches employ column chromatography. Techniques 1,2 and 3 utilise ion exchange chromatography (DEAE-Sephacel) whereas techniques 4 and 5 use hydrophobic chromatography (phenyl sepharose). The following sections provide a brief description of the approaches.

3.1.1 Method 1

This procedure was employed by Koohmaraie (1990b) in a paper designed to compare hydrophobic and ion exchange protocols for the extraction of the calpain system components. The key points about this protocol are that tissue samples were prepared in a relatively small volume of extraction buffer (3 sample volumes) containing buffering and Ca^{2+} chelating agents. Additionally, cysteine residues at the active site of the calpain protease oxidise to form intra or intermolecular disulphide bonds rendering the protease inactive. However, ß-Mercaptoethanol reduces the disulphide bond and reinstate the original cysteine structures by undergoing oxidation itself. The sample was then diluted to lower its ionic strength and enhance protein/bead association, before being gradually poured onto the top of the DEAE column. A **linear gradient** of NaCl gradually elutes all proteins binding with a strength of less than 0.4M NaCl. This includes calpains and their inhibitor.

3.1.2 Method 2

This approach has been employed by Wheeler and Koohmaraie (1991a) in a set of experiments intended to extract and measure both the calpain and lysosomal protease systems from skeletal muscle. Differences from Method 1 include the use of more extraction buffer for sample homogenisation (10 sample volumes) which dilutes the sample and increases the ratio if Ca^{2+} chelating agents to Ca^{2+}. Furthermore, the extraction buffer uses sodium acetate as buffering agent and includes detergent (Triton X-100). Sodium acetate is included because the ultimate Ph of the buffer (5.8) falls beyond the range of TRIS but within its effective buffering range. Triton X-100 disrupts the lipid bilayer component of the sample thus releasing membrane bound proteins, and lysosomal cathepsins. This follows the suggestion that calpains are membrane associated prior to

their activation *in vivo* (Gopalakrishna *et al.*, 1986; Melloni *et al.*, 1991). The dilution step of Method 1 is replaced with dialysis which has the same effect as the dilution ie. lowers the conductivity of the sample. Dialysis also removes small molecular weight proteins, salts and lipids ie. performs a crude purification. If several samples are to be analyzed and compared dialysis in the same buffer will equate sample compositions and variables, such as salt content, which could influence the binding behaviour of the proteins, can be removed. The elution protocol has been modified from a linear gradient to a **linear gradient/bulk elution** combination. Initial fractions are generated from a linear gradient running from 25-200mM NaCl. This is important because calpastatin and calpain I elute at similar NaCl concentrations and a shallow slope will encourage better separation. The jump to higher NaCl will elute all the calpain system components remaining (ie., calpain II). Furthermore, this bulk wash concentrates calpain II and reduces the time and number of fractions collected.

3.1.3 Method 3

This strategy employs DEAE-Sephacel, and was used to determine the effects of nutrition on the calpains (Thomson *et al.*, 1992). In this method there was no attempt to alter the conductivity of the sample either through dilution or dialysis and the sample was loaded directly onto a small column following a filtration step. The elution step is a **bulk wash** process. Single column volume washes of equilibration buffer with 100, 200 and 400mM NaCl successively remove calpastatin, calpain I and calpain II from the column.

3.1.4 Method 4

This protocol was first described by Gopalakrishna *et al.*, (1985) and was investigated as an isolation procedure for calpains. Hydrophobic interaction chromatography using phenyl sepharose was used. The specific protocol followed in this work was the "routine isolation" described by Golpalakrishna *et al.*, (1985). The sample was homogenised in 5 sample volumes of extraction buffer including a potent acid protease inhibitor pepstatin A (Utterhaegen *et al.*, 1994). Following sample centrifugation and filtration the matrix is added. Phenyl sepharose beads had previously been washed in buffer containing 0.25M NaCl and leupeptin. Leupeptin is a transition-state-analogue inhibitor of cysteine protease (Koohmaraie 1992a) and following the activation and conformational change of the calpain molecule, it binds to and inhibits the protease in order to prevent premature autoproteolysis. Elution is a **bulk process** designed to sequentially remove

ıgredients used in the binding process.

3.1.5 Method 5

his technique is similar to the routine isolation, however, this method proceeds without any ıhibitors (ie., Pepstatin A or Leupeptin). The inclusion of inhibitors may mask the activity of the roteases, and the tactics intended to remove them from the protein fraction may not be effective. he protocol is relatively swift, uncontrolled proteolysis should be kept to a minimum.

.2 Methods

3.2.1 Extraction methodology

efer to appendices 1-5 for methods, for detailed descriptions refer to the original papers.

3.2.2 Assay for enzyme activity

ıe activities of calpain I and II were determined using casein as a substrate. This procedure :termines the extent of proteolysis by measuring the peptides released from casein ie., the increase A_{278} absorbance in the soluble fraction after treatment with trichloroacetic acid (TCA). 0.5ml ɔm fractions were incubated with 0.5ml Ca^{2+} casein assay media (10mM TRIS-HCl, 5mM $CaCl_2$, nM NaN_3, 5mg/ml casein, 10mM ß-MCE, pH 7.5) for 60 minutes at 25°C. The reaction was ɔpped by adding 1.0ml of 5% trichloroacetic acid (ice cold). The assay mixture was then ntrifuged at 4000rpm for 15 minutes to remove the precipitate, and the absorbance of the pernatant was measured at 278nm. Ca^{2+} independent protein hydrolysis was determined in the me manner using EGTA containing casein assay media (10mM TRIS-HCl, 10mM EGTA, 1mM ıN$_3$, 5mg/ml casein, 10mM ß-MCE, pH 7.5). Each fraction was assayed in duplicate with each ɜay media.

ılpastatin was assayed for by adding 0.5ml of supernatant to partially purified sheep calpain II 5 Units/gm). Following incubation with 0.5ml Ca^{2+} and EGTA casein assay media for 1 hour at °C the reaction was terminated by addition of 1ml 5% TCA. Calpastatin activity was determined the amount by which the added calpain II is reduced.
ternatively, calpastatin was determined by following the heated procedure, or boiling technique

(Shackelford *et al.*, 1993). Aliquots of the original homogenised sample were heated in a water bath for 15 minutes before being placed in an ice bath for 5 minutes. The resulting coagulated protein was scrambled with a glass rod to facilitate separation of the supernatant and pellet during centrifugation at 4000rpm for 15 minutes. Samples from the supernatant were then assayed for inhibitory activity as described above.

Total yield of each protease was determined by the following equations developed by Koohmaraie (personal communication), and survey of the literature (Koohmaraie 1990b; Wheeler *et al.*, 1991a; Ilian *et al.*, 1992). One unit of calpain activity is defined as the amount of enzyme that catalyses an increase of 1.0 absorbance unit at 278nm in 60min at 25°C.

Equation 1:

(No. Fractions x Average Activity/ml x Fraction Size) / Sample size = Units/gm

- *No. Fractions* refers to the number of fractions with observed activity.
- *Fraction size* denotes the volume of the fractions collected.
- *Sample size* describes the weight of the original tissue sample, this is required to convert the units figure into units/gm.
- *Average activity/ml* reports the calculated activity (abs 278nm) contained in 1ml of fraction volume. The definition of this is described in Equation 2.

Equation 2:

(Average activity x Correction Factor) = Average Activity/ml

- *Average activity* is the sum of the activities of the fractions displaying calpain activity divided by the number of fractions displaying activity.
- The *correction factor* refers to the factor by which the average activity must be multiplied in order to determine the activity of 1ml of fraction. For example if only 0.5ml of fraction is assayed, and the total assay volume after addition of TCA is 4ml, then the dilution factor is (4/0.5) = 8. It is important to calculate this factor because each method has different assay volumes, and correction of the activities to a common factor must be made to compare results.

Convert the figures obtained from negative calpain activity to positive inhibition by subtracting observed activity from added activity in the fractions.

Equation 3:

Control Activity - Fraction Activity = Positive inhibition absorbance

3.3 Results

Because of the variety of approaches used in this chapter to extract and analyze the calpains and calpastatin, it was easiest to subdivide this section into five subsections presenting the results from each methodology. A comparison of results is presented in the results summary.

3.3.1 Method 1 Results

A total of sixty-four 3ml fractions was collected from the DEAE Sephacel column after application of a linear gradient of 0-400mM NaCl. The protease activities of every second fraction are shown in Figure 3.1. Results from the assay for calpastatin are in Figure 3.2.

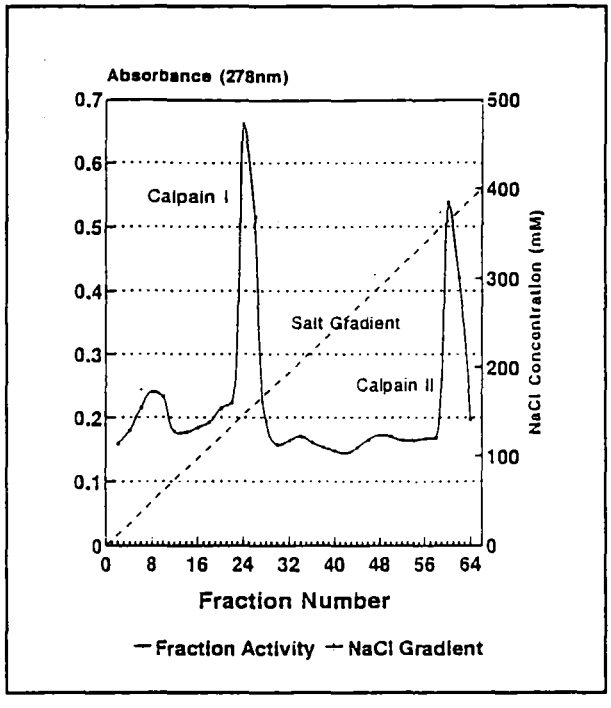

Figure 3.1 Method 1, calpain I and II. Graph showing the change in Absorbance (278nm) following elution and a 1 hour standard incubation. NaCl inflow concentration also shown.

Figure 3.1 demonstrates the presence of calpain I and II in fractions 24-26 and 60-62 respectively. The elution profiles of these peaks bear some similarity with to other in terms of height and width. The proteases elute at ionic strengths of 125mM for calpain I and 350mM for calpain II. This was determined by approximating the position on the gradient line at which the calpains began eluting, and then extrapolating this position across to obtain an ionic value. The relatively large ionic difference suggests that a clean separation has taken place.

The equation described under section 3.2.2 require variables determined by the experiment protocol for calculation of enzyme activity. Table 3.1 presents these variables for Method 1.

	Calpain I	Calpain II
No. Fractions	2	2
Fraction Size (ml)	3	3
Sample Size (gm)	5	5
Correction Factor	4	8
Average Activity (278nm)	0.59	0.48
Units/gm	2.83	4.61

Table 3.1 Calculation of units/gm calpain I and II from Method 1.

It is apparent from this calculation that this method has been successful in separating and quantifying both proteases. One unit of calpastatin is the amount of inhibitor that completely suppresses one unit of calpain II. A volume of calpain (with known activity) is added to the assay tube and reduction in this activity is interpreted as calpastatin activity. The activity of the calpain II used in this assay was 0.187 (abs 278nm), this is displayed in Figure 3.2 as a straight line.

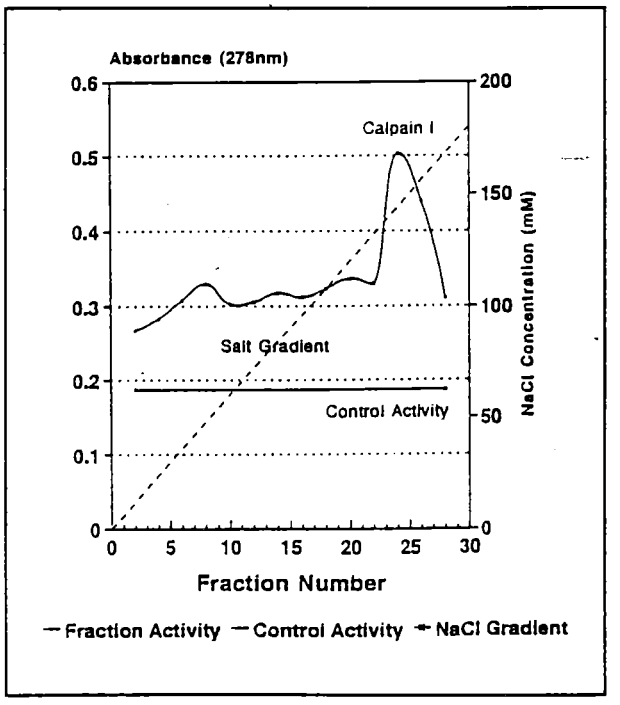

Figure 3.2 Method 1 calpastatin. Graph showing the change in A_{278} of fractions (eluted from a DEAE column) after incubation for 1 hour under standard assay conditions. Control describes the activity of calpain II added to each tube and is represented by a horizontal line. The NaCl concentration at the column inflow is also shown.

It is evident that at no point in this assay did the activity of the fractions drop beneath the 0.187 threshold, this indicates that there is no measurable calpastatin activity. A possible reason for this is that the assays used made no effort to account for the intrinsic activity within each fraction that results from the presence of non-calpain proteins. Therefore calpastatin may have been present in the assayed fractions, but because non-calpain proteolytic proteases were contributing to the total A_{278} measurement in these fractions the inhibition could not be distinguished.

The protein profile displayed in Figure 3.3 indicates a significant quantity of protein elutes from the column and is captured by the early fraction collection tubes.

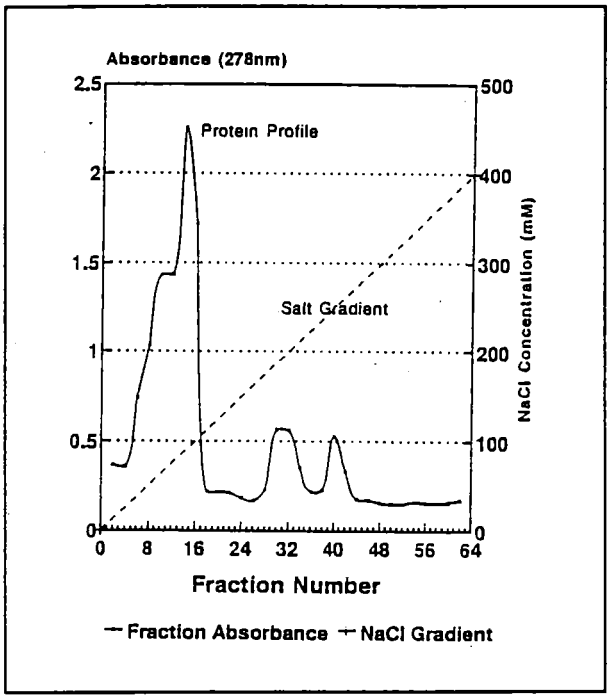

Figure 3.3 Method 1 protein profile. Graph showing the A_{278} absorbance of fractions eluted off the DEAE Sephacel.

3.3.1.1 Method 1 discussion

Calpains I and II have been successfully extracted and quantified by this procedure, however this work has failed to determine calpastatin. The protein content of fractions collected in this method contributes to the total absorbance A_{278} figure (Figure 3.3), and without the means to differentiate how much of the measured absorbance is calpain and how much is miscellaneous protein, it is impossible to ascertain if the calpain II added to fractions in figure 3.2 has been inhibited. For

example, fraction 10 may contain calpastatin and may have significantly inhibited the 0.187 units of calpain added. However, it may also contain high concentrations of protein absorbing at 278nm. This protein would contribute to the overall observed absorbance and prevent the detection of inhibition. This problem could be overcome by measuring the absorbance of each assayed fraction in the presence of calpain II and EDTA. The EDTA would ensure that the calpain II remained inactive and the activity of this fraction following a normal incubation period would reflect non-calpain dependent absorbance. This could be accounted for in a simultaneous assay in which Ca^{2+} replaced EDTA, thus encouraging calpain activity.

An interesting observation from Figure 3.2 is that the peak found between fractions 22 and 28 corresponds to the calpain I peak seen in Figure 3.1. However, at this time the peak is approximately 2/3 its original height. This probably originates because the calpastatin assay was performed approximately 24 hours after the original calpain assays. In this time, calpain I may have undergone autoproteolysis and reduced itself. This time dependent behaviour will be further examined in chapter 5.

A final observation is that the protein peaks shown in Figure 3.3 do not coincide with the peaks of activity in Figure 3.1. This means that the location of the protease peaks cannot be approximated by examination of the protein profile.

3.3.2 Method 2 Results

In this method seventy-two fractions with a volume of 3ml each were collected using a linear gradient/bulk wash combination. Protease activities from every second fraction are shown in Figure 3.4 and calpastatin data is displayed in Figure 3.5.

Figure 3.4 is similar to Figure 3.2 in that it shows clearly the separation and presence of two peaks of proteolytic activity. Calpain I was eluted at an ionic strength of approximately 75mM NaCl, while calpain II was eluted with a wash of 400mM NaCl. The shape of the calpain I curve was similar in both Figures 3.1 and 3.4, calpain II curve is noticeably shorter and broader in Figure 3.4 than the peak in Figure 3.2.

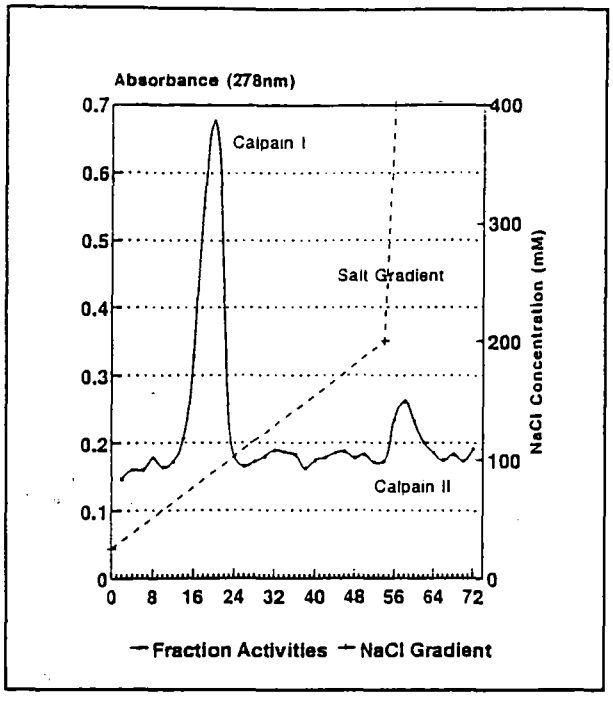

Figure 3.4. Method 2 calpain I and II. Graph displaying the A_{278} of fractions incubated for 1 hour following standard assay conditions. Inflow NaCl concentration is also included.

For a protein to elute off an ion exchange column a specific ionic strength must be reached. In Method 1 there is a gradual increase in the ionic strength of the elution buffer, consequently calpain II will remain bound until such time as the required elution concentration is reached. At this time, however, the protein will elute and, since the ionic strength of the column is relatively homogenous (and there are no pockets of differential ionic concentration) the protein will elute cleanly. In the second method, however, a strong 400mM wash is applied to the column. This will elute calpain II but because the column is formerly at 200mM NaCl (following the gradient elution), the actual ionic strength throughout the column will become heterogenous. This could lead to variable elution of calpain II and a broader shorter peak of activity. The total yields of calpain I and II are determined by equations 1 and 2 and are 3.73 units/gm for calpain I and 4.46 units/gm for calpain II. Total yield (U/g) of protease from Method 1 and 2 are similar. This will be discussed in Results Summary.

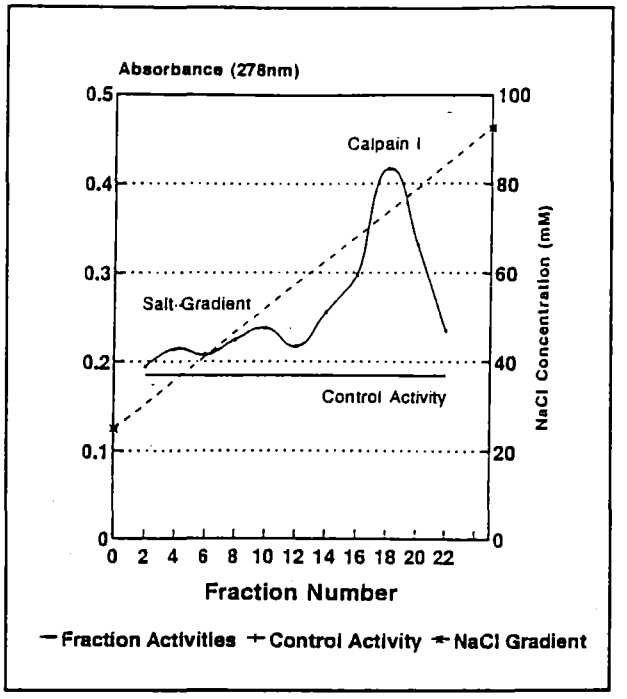

Figure 3.5 Method 2 calpastatin. Graph displaying A_{278} increase following fraction incubation for 1 hour under standard assay conditions. Calpain II activity is displayed as a horizontal control line, inflow NaCl concentration is also shown.

3.3.2.1 Method 2 discussion

Calpastatin was not successfully extracted in this experiment. At no point in the survey of tubes shown in figure 3.5 does the activity of the fractions drop below the added calpain II activity. The reduction in calpain I activity over time, observed in Method 1 appears to be confirmed by this assay. Again the calpain I activity has decreased in, what appears to be, a time dependent manner. In conclusion, this method is successful in separating and assaying calpains I and II but not calpastatin. Although the failure to detect calpastatin probably results from a failure with the assay system rather than the separation.

3.3.3 Method 3 Results

Method 3 generated a total of 18 fractions with 5ml volumes. The first 5 of these fractions are the result of a 100mM NaCl wash intended to elute calpastatin. The next five arise from a 200mM wash to remove calpain I and the remaining 5 fractions represent a 400mM wash which should contain calpain II. Figure 3.6 contains the data from calpain assays, and Figure 3.7 include data from the calpastatin assay.

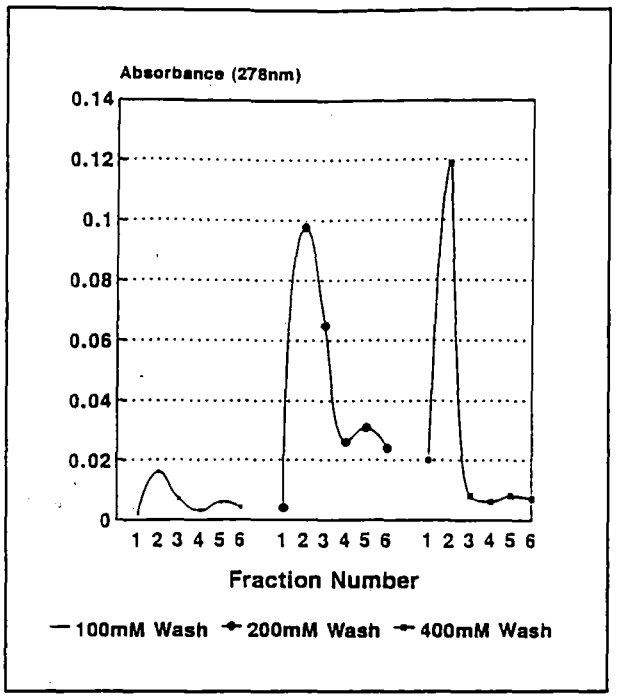

Figure 3.6 Method 3 calpain I and II. Graph showing the increase in A_{278} of fractions (eluted from a DEAE column in a bulk wash) incubated for 1 hour under standard assay conditions.

It is clear from Figure 3.6 that the fractions eluted with 100mM NaCl contain insignificant proteolytic activity (ie., A_{278} = 0.018). This was anticipated since the 100mM wash is designed to elute calpastatin. Fraction 2 and 3 from the 200mM wash and fraction 2 from the 400mM wash do, however, display calpain activity. It is likely that the former fractions contain calpain I and the latter calpain II.

Because of the nature of the elution (ie. column volume washes) the activity is limited to just one or two fractions. A total replacement of the column fluid takes place with each wash step and is collected separately. Proteins should elute promptly at each NaCl concentration. Calculation of the calpain activities reveals that 2.43 Units/gm calpain I and 4.76 Units/gm calpain II were extracted. These activities are not dissimilar to those observed in Methods 1 and 2. This reinforces the idea that the DEAE Sephacel based methods of separation are successful in eluting all the calpain protease from skeletal muscle samples.

Manifest in Figure 3.7 is a trough of activity across fractions 2,3,4 and 5. Because the activity of these fractions drops below the activity of the added calpain II (0.063), the fall represents a positive inhibition and may the presence of calpastatin over this region.

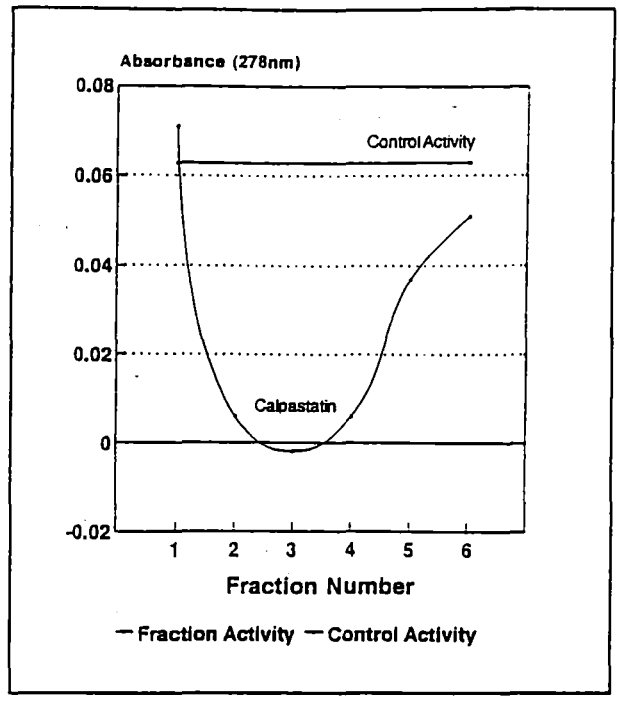

Figure 3.7 Method 3 calpastatin. Graph showing increase in A_{278} of fractions from method 3's 100mM NaCl wash when assayed under standard conditions. Basal calpain II activity (control) is shown as a horizontal line.

Calculation of Units/gm calpastatin yielded from this method was 5.18.

3.3.3.1 Method 3 discussion

These results provide evidence that both calpains and calpastatin have been successfully separated from the tissue sample. These results differ from the previous approaches where only calpain I and II were able to be measured accurately.

3.3.4 Method 4 Results

In these experiments a total of 6 fractions only were collected. The first fraction (1.5ml) was composed of the drain that passed through the column upon application of the sample, which for the purposes of this discussion has been called Fraction 0. Fractions 1, 2, and 3 (all 2ml volumes) represent various wash phases designed to remove inhibitors and elements contributing to the association of bead and protein. Fraction 4 (4ml) elutes calpain II, fraction 5 (2ml) elutes calpain I. The activities from each of the fractions are in Table 3.2 and Figure 3.8. Calpastatin was determined by a boiling technique which required no fractionation (Shackelford *et al.*, 1993).

Fraction	Absorbance
Void Volume (1)	0.016
2ml BufferA + .25M NaCl (2)	0.007
2ml Buffer A (3)	0.008
2ml Buffer A - Leupeptin (4)	0.004
4ml Buffer B + 0.1M NaCl (5)	0.004
2ml Buffer B (6)	0.013

Table 3.2 Method 4 protease assay. Table showing the A_{278} increase apparent in fractions incubated for 1 hour under assay conditions. The numbers in parentheses are the numbers denoting each fraction in the graph.

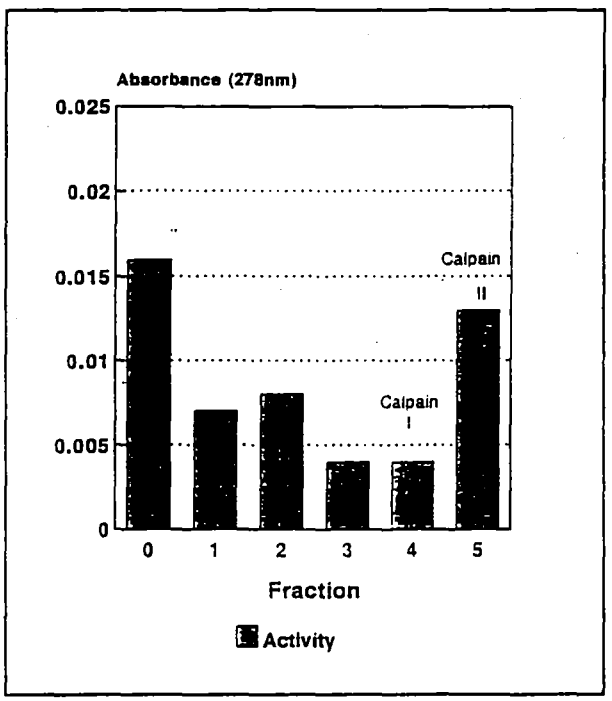

Figure 3.8 Method 4 protease assay. Graph showing A_{278} increase seen in fractions incubated for 1 hour under standard assay conditions.

Figure 3.8 shows that there is activity in all fractions including the void volume which indicates that a problem exists in protein/bead association. This distribution of activity means that calpain I and II are not cleanly eluted in fractions 4 and 5, but unselectively, at each step. It is perplexing as to why this should be the case and why calpain activity is detectable in fractions 0-2 which theoretically should contain the calpain inhibitors pepstatin A and leupeptin. Quantification of the calpain fractions based on the activities of the fractions which theoretically contain calpain I and II (ie., 1,5 and 6) is 0.52 units/gm for calpain I and 1.28 units/gm for calpain II.

The technique has yielded significantly smaller quantities of calpain I and II (per gram) when compared to Methods 1-3 despite the initial source of material being the same. Calpastatin activity as directly assessed after boiling to remove proteases, the units/gm yield of calpastatin was 4.02.

3.3.4.1 Method 4 discussion

Calpains were not effectively localised by this procedure, and their yields were significantly reduced when compared to methods 1-3. The proteolytic activity apparent in the void volume is difficult to explain since any proteases in the sample should be inactive due to the presence of pepstatin and leupeptin. More work is required to assess this point. The calpastatin activity is similar but less than that obtained from Method 3. In conclusion, this technique has successfully separated calpain I, II and calpastatin from muscle tissue. However, the separation of the proteases does not appear to limit them exclusively to their expected locations of fractions 5 & 6.

3.3.5 Method 5 Results

The experimental design of this method is similar to that of method 4, but excludes all protease inhibitors. Table 3.3 contains the activities within each fraction, which are graphically represented in Figure 3.9. The calpastatin data is included in the text.

Fraction	Absorbance
Drain (1)	0.013
2ml BufferA + .25M NaCl (2)	0.012
2ml Buffer A (3)	0.008
2ml Buffer A (4)	0.013
4ml Buffer B + 0.1M NaCl (5)	0.021
2ml Buffer B (6)	0.019

Table 3.3 Method 5 protease assay. Table showing A_{278} increase in all fractions incubated for 1 hour under standard assay conditions.

Figure 3.9 illustrates that the fractions intended to contain calpains I and II have a greater activity than any other fraction. This differs from Figure 3.8 where the drain fraction was significantly greater than both calpain I and II activities, indicating a more selective separation has taken place. However, the problem still remains that the appearance of calpain activity is not limited to fractions

4 and 5 - rather it is spread over all fractions with a slight greater activity in fractions 4 and 5.

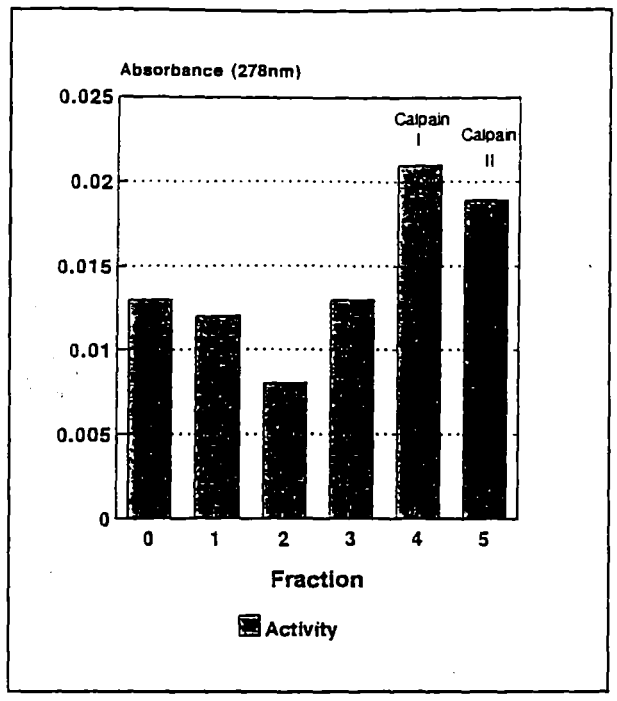

Figure 3.9 Method 5 protease assay. Graph of data showing the A_{278} increase observed in fractions incubated for 1 hour under assay conditions.

Again this technique has yielded significantly smaller quantities of calpain I and II compared to the ion exchange methods. 0.76 units/gm calpain I and 6.72 units/gm of calpain II were extracted. The calpastatin assay follows the protocol from Method 4 and produced 3.96 units/gm of enzyme.

3.3.5.1 Method 5 discussion

Overall, the calpain activities are higher than those observed in Method 5 probably because of the lack of protease inhibitors. If this is the case then it is concerning to use such inhibitors for fear of compromising accurate quantification of the calpains. Again the boiling technique used to isolate calpastatin has been successful. Calpastatin activity is almost identical to that obtained in previous experiments, this lends confidence to its reproducibility and reliability. In conclusion, this method unselectively separates calpain I and II from muscle and calpastatin activity is measured satisfactorily by the boiling technique.

3.4 Conclusion and Discussion

In general, the DEAE-Sephacel based methods of extraction are more successful in the separating calpain I, II and calpastatin compared to the hydrophobic protocol. This conclusion is based on the observation that discrete peaks of protease and inhibitor activities are observed in Figures 3.1, 3.4, and 3.6. This differs from hydrophobic chromatography where all the fractions appear to contain greater or less proteolytic activities. The inability of methods 1 and 2 to separate calpastatin successfully is probably a problem that can be solved by further experimentation. The method of assaying calpastatin in addition to the extraction method also needs further evaluation. For example, the current inability to detect the calpastatin component can be solved by using the boiled quantification procedures employed in methods 4 and 5.

Technique	System Component	Units/gm
Method 1	Calpain I	2.83
	Calpain II	9.22
	Calpastatin	0.00
Method 2	Calpain I	3.73
	Calpain II	8.91
	Calpastatin	0.00
Method 3	Calpain I	2.43
	Calpain II	9.52
	Calpastatin	5.18
Method 4	Calpain I	0.52
	Calpain II	1.28
	Calpastatin	4.02
Method 5	Calpain I	0.76
	Calpain II	6.72
	Calpastatin	3.96

Table 3.4 Table showing the total yield of calpain system components acquired from each of the five methods assessed in this chapter.

A comparison of the total yield of the enzymes and the reproducibility of their yield is shown in Table 3.4. Techniques using DEAE are usually more consistent in separating calpain I and II. One

possible difficulty with DEAE is its ability to separate calpastatin. Hydrophobic chromatography protocols showed significantly lower yields of calpain I and method 4 gave very low levels of calpain II activity. The conclusion that ion exchange is superior to hydrophobic chromatography agrees with the results of Koohmaraie (1990b). Calpastatin figures from methods 4 and 5 are very similar. This is because the extraction procedures were identical and were unrelated to the chromatography techniques employed. In other words, DEAE based methods of extraction yield consistently higher activities. An accurate and reliable yield of calpastatin by DEAE requires further investigation. Phenyl sepharose chromatographic methods gave considerably more variable yields of calpain I and II, and the activities were substantially less than those obtained by the DEAE methods.

With respect to time of analysis, the protocols using small column volumes and bulk elution technique are superior. Specifically, Methods 3, 4 and 5. The entire extraction procedure from these approaches takes approximately 2 hours compared to 10 hours or more by the long column and gradient elution techniques. Time is important since the proteins of interest have a limited life span due to autoproteolysis. Consequently, the proteases must be measured promptly to obtain accurate and representative quantification. In addition, the nature of future work demands that a number of samples be compared simultaneously. Equipment limitation in itself restricts the number of samples that may be analyzed. Fast analytical procedures would alleviate the severity of this constraint, providing the proteases and inhibitor can be separated and analyzed reproducibly.

The final point worthy of consideration is the ease of extraction. This is a purely subjective comparison and is not supported by any measured parameters. Up until the column stage (ie the preparation, homogenisation, centrifugation) the procedures are very similar. However following the loading of the columns differences in the ease of the procedures can be observed. In Methods 1 and 2 after loading of the columns little attention is required until the assay procedures. Methods 3, 4 and 5, however, demand constant experimental overseeing since they rely on differential bulk elutions. Thus, the ease and speed are oppositely ranked ie., the fastest are also the most labour intensive and the slowest are the least. This is particularly important if a large number of samples are to be processed.

In summary the DEAE protocols appear superior to the phenyl sepharose based extraction procedures. This conclusion is based on the yield and relative success of separating calpain I, calpain II and calpastatin. The fastest DEAE protocol is Method 3 but this process is also the most labour intensive. The phenyl sepharose protocols are fast but are unable to consistently separate calpain I, II and calpastatin at this time.

Chapter 4

Development of an extraction and measurement technique suitable for the detection of the calpains and calpastatin.

4.1 Introduction

This project required reliable, reproducible and accurate quantification of the enzymes in the calcium dependant proteolytic system. A crude protein preparation is satisfactory but a complete separation and quantification of the individual enzymes (calpains and calpastatin) is required. In agreement with Koohmaraie (1990b), chapter 3 reported that the DEAE protocols are superior to the phenyl sepharose chromatography in achieving the separations. This conclusion was based upon the yields and achievement of the chromatographic separation using DEAE-Sephacel. The calpastatin component can be quantified by the boiling procedure.

This chapter describes an experimental protocol intended to be used in subsequent experiments. It is based upon the Method 1 protocol in Chapter 3 with some modifications. Method 1 protocol was selected over the swifter Method 3 because it appeared to be more successful in reliably separating calpains I and II. Two discrete peaks of proteolytic activity were seen using Method 1, while some doubts exist as to whether the bulk elution ionic strengths (ie., 100mM, 200mM and 400mM) were separating the components accurately and completely. Additionally this chapter will illustrate the reproducabilty of the technique, and results will be compared with those from other workers.

4.2 Methods

4.2.1 Animals

A total of 10 ewes (½ Merino x ½ Texel, 15 months old, 50-60kg liveweight) were killed on the same day at a local meat processing plant.

4.2.2 Calpain preparation and assay

Calpains were prepared from a 10gm sample of *longissimus dorsi* muscle taken 20 minutes after death and prior to electrical stimulation. A 5gm subsample of the muscle was trimmed of visible fat and connective tissue, minced with scissors and a scalpel, and homogenised in 5 vols (25ml) of cold extraction buffer (100mM TRIS-HCL, 10mM ß-MCE, 10mM EDTA, pH 8.3) using a polytron homogeniser set on high speed. Three 30 second homogenisations were separated by cooling periods of 30 seconds. The homogenate was centrifuged at 15,000 rpm for 1 hour at 4°C, the supernatant was decanted off and filtered through a washed glass wool/cheese cloth sandwich. While stirring gently, the pH of the filtered extract was adjusted to 7.5 with 1M acetic acid and then dialysed for 18 hours against dialysis buffer (40mM TRIS-HCL, 3mM EDTA, 10mM ß-MCE, pH 7.5) at 4°C. The extract was filtered through a washed, moist glass wool/cheese cloth sandwich and then loaded onto a DEAE-sephacel column (1.5 x 20cm) which had been equilibrated with elution buffer (40mM TRIS-HCL, 0.5mM EDTA, 10mM ß-MCE, pH 7.5). The column was washed with 250ml elution buffer and the bound proteins eluted by a continuous gradient of NaCl from 25mM to 350mM (120ml of each) in elution buffer.

The activities of calpain I and II were determined using casein as a substrate as described in chapter 3. Briefly, 0.5ml of fractions 1-55 and 0.25ml of fractions 56-80 were combined with appropriate volumes of Ca^{2+} and EGTA casein (10mM TRIS-HCL, 1mM NaN_3, 10mM ß-MCE, 5mg/ml Casein, 5mM $CaCl_2$ or 10mM EGTA, pH 7.5) to bring the total volume up to 1.0ml. The mixture was incubated at 25°C for 1 hour, the reaction stopped by the addition of 1.0ml ice cold 5% TCA. Assay tubes were spun at 4000rpm for 15 min and the soluble digestion products in the supernatant measured spectrophotomerically at 278nm. One unit of calpain activity was defined as the amount of enzyme catalysing an increase of 1 absorbance unit at 278nm in 60min at 25°C.

4.2.3 Calpastatin preparation and assay

Calpastatin was extracted from the sample following the centrifugation step described above. A 1ml subsample of the extract was placed in a 95°C water bath for 15min before being placed in ice. The sample was stirred with a glass rod to disperse the coagulated protein, and then spun at 11000rmp for 5mins. The resulting supernatant was assayed for calpastatin by incubating a 250µl subsample of the soluble calpastatin fraction with a volume of calpain II possessing an activity of 0.25-0.30. Calpastatin activity is determined by the loss of activity in these tubes.

4.2.4 pH and Tenderness measurements

Carcasses were aged for 24 hours (at 12°C) before loin chops were removed under normal industry boning conditions. Chops were vacuum packed and aged up to 2 weeks at 4°C. At this time tenderness was assessed using a MIRINZ tenderometer according to standard techniques (Quality Assurance for Tenderness May 1993 MIRINZ technical report 872, ISSN 0465-4390). Briefly, loin chops are trimmed to a uniform depth (40mm laterally) before being cooked in a thick walled plastic bag submerged in a water bath at 90°C. On reaching an internal temperature of 80°C, the loins are immersed in an ice bath. Randomly chosen samples of a uniform size (2cm x 1cm x 1cm) were sampled from the cooked loin. The 2cm length of the sample ran parallel to the muscle fibres. The section was placed in the Tenderometer and the force (Kpa) required to sever the tissue across the fibres recorded. Kpa was converted to kilograms force (Kgf) by Equation 4:

Equation 4

$$Kgf = (0.20116 \times Kpa) - 1.1373$$

pH and temperature were recorded at intervals prior to boning, and again at 2 weeks. This was done using a Hanna HI9025C portable meter with an Orion Ross combination spear tip pH electrode (81-63) and temperature probe. pH was measured in the LD muscle. Care was taken to ensure sampling was within a limited region of the muscle. The pH probe was standardised at sampling temperatures, and cleaned to remove any residual fat collected between individual sampling times.

4.2.5 Statistical analysis

Data was tabulated in Quattro Pro spreadsheets, statistical analysis used Minitab Statistical software package. Data was analyzed using ANOVA, Students *t* Test, and regression.

4.3 Results

4.3.1 Calpain system components

Animal	Calpain I	Calpain II	Calpastatin
1	0.05	0.41	2.66
2	0.39	0.28	3.11
3	0.19	0.40	3.69
4	0.42	0.34	3.73
5	0.57	0.16	3.35
6	0.47	0.70	3.11
7	0.24	0.49	2.43
8	0.36	1.03	3.62
9	0.16	0.36	3.29
10	0.14	0.57	3.23
Average	0.30 (±0.17)	0.47 (±0.25)	3.22 (±0.43)

Table 4.1 Calpain I, II and calpastatin activities (units/gm) in LD muscle 20 min after death.

4.3.2 Temperature, Tenderness and pH results

Figures 4.1, and 4.2 illustrate the average changes in temperature and pH, in LD muscles from 10 animals over a 24 hour commercialised chilling regime. Table 4.2 presents the tenderness of loin chops at 2 weeks of age.

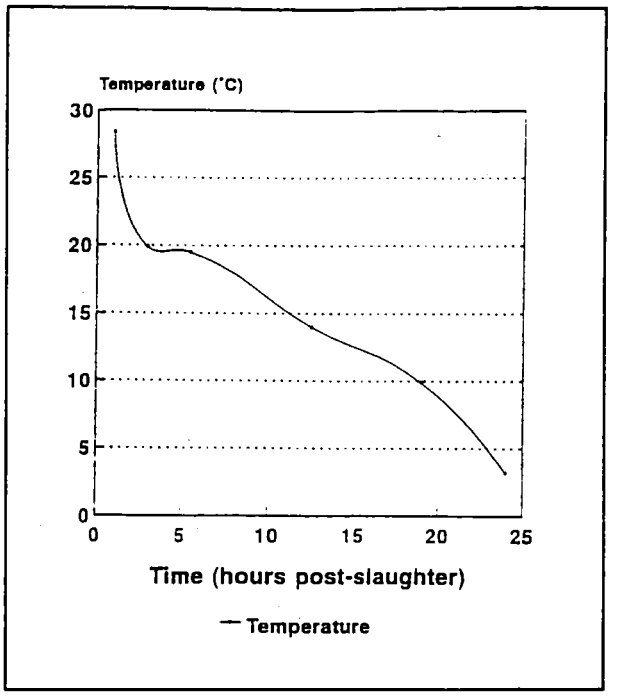

Figure 4.1 Temperature profile of the LD muscle stored under standard industry conditions up to 24 hours post-slaughter.

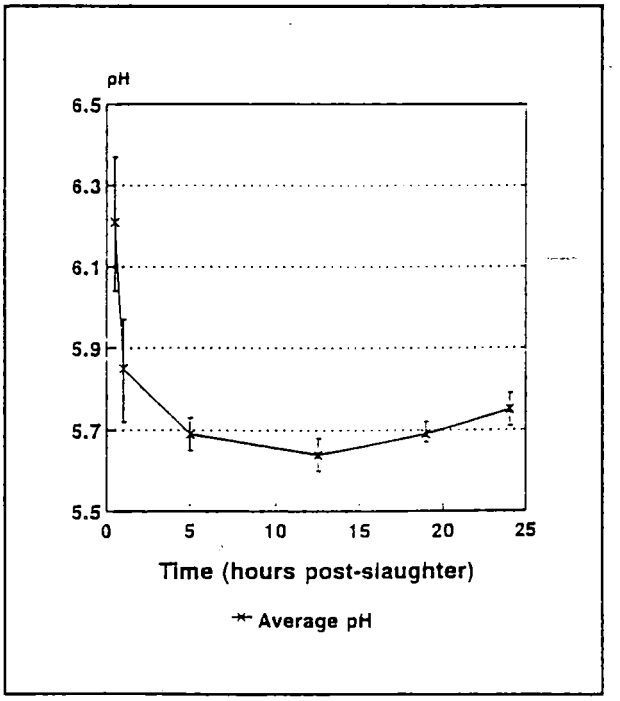

Figure 4.2 pH profile of the LD muscle stored up to 24 hours post-slaughter under industry cooling conditions.

In Figures 4.1 and 4.2 like reductions in temperature and pH are apparent. Final pH is reached after 12 hours and final temperature is attained 24-48 hours post-slaughter.

Animal	Tenderness (Kgf)
1	8.71
2	10.29
3	8.40
4	8.66
5	10.93
6	11.31
7	4.29
8	8.79
9	7.55
10	16.22
Average	9.51 (±3.1)

Table 4.2 Tenderness values obtained for loin (LD) muscle after 2 weeks ageing. Values are expressed in Kgf, and are the average of between 4 and 8 individual samples.

Figure 4.3 shows the correlation of calpain I, II and calpastatin with tenderness within each animal.

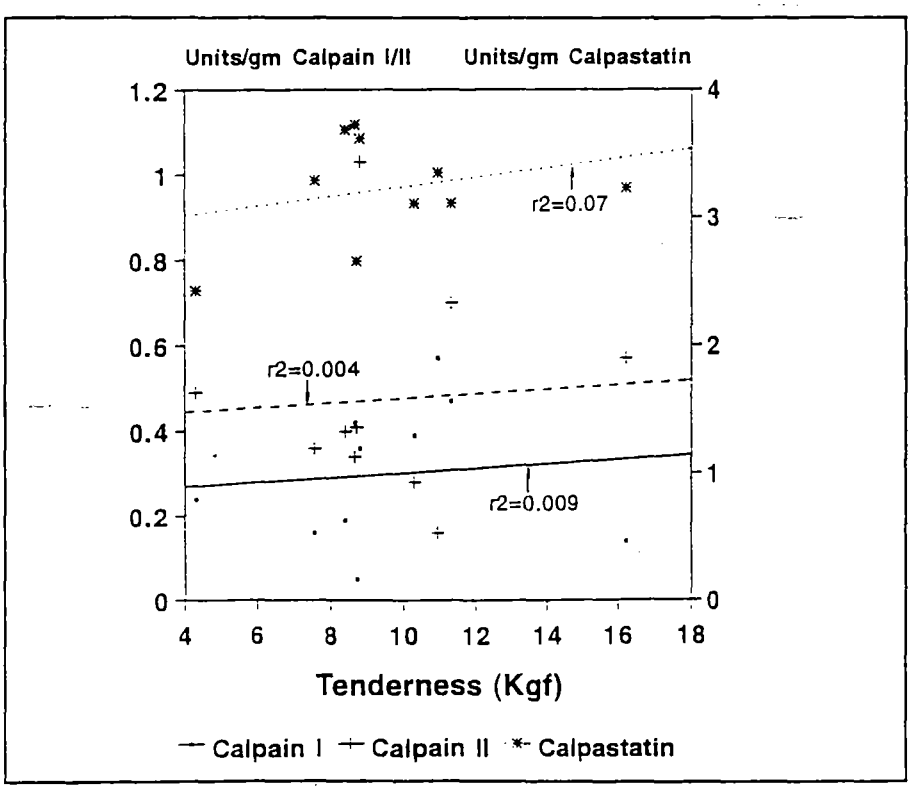

Figure 4.3 Graph illustrating correlation between enzymes (units/gm) and tenderness in LD muscle samples.

It is apparent from this figure is that there is considerable scattering of both enzyme activities and tenderness. This is supported by the r^2 values which are not significant. There appears to be, however, positive correlation between the enzymes (calpain I, II and calpastatin) and toughness.

4.4 Conclusion and Discussion

4.4.1 Calpains and Calpastatin

The technique employed in these experiments has been successful in extracting calpains I and calpain II and calpastatin from LD muscle. Activity profiles of the enzymes show there is no overlap of calpain I and II (data not shown). However, the yields of enzyme (units/gm) seen here are noticeably less than those determined by DEAE-Sephacel in Chapter 3 (Methods 1 and 2). A survey of current literature reveals the following trends.

Calpain I	Calpain II	Calpastatin	Source
Ovine			
0.76	1.15	1.25	Koohmaraie 1990a
0.58	0.67	1.25	Kretchmar *et al.*, 1990
1.23	1.39	2.43	Koohmaraie *et al.*, 1991
Porcine			
1.18	0.93	1.49	Koohmaraie *et al.*, 1991
Bovine			
1.09	1.12	4.13	Koohmaraie *et al.*, 1991
1.32	0.81	3.28	Morgan *et al.*, 1993
1.05	1.44	3.72	Wheeler *et al.*, 1992

Table 4.3 Summary of literature presenting enzyme yields (Units/gm) measured by the same assay protocol used in this work, and at similar times, from LD muscles.

There would appear to be similarity in the levels of enzymes from these various investigations. Calpain I and II exist in a roughly even ratio of 1 unit/gm, while calpastatin levels fluctuate from less than 1 to 4. The experiments in this thesis reflect these trends, although the absolute yields of protease are less than those reported in Table 4.3. This could be due to variation in the sampled material (eg., animals) or experimental techniques (eg., extraction or assay). However, it seems doubtful that disparity is produced by the animals since the above table lists enzyme yields from three different species and yet the values are similar. It is more likely that experimental protocols

vary in some way.

Furthermore, it is not obvious why the protease activities in this chapter should be less than those reported in Chapter 3. The extraction method has been altered slightly (eg extraction buffer volume, gradient volumes) but not in a manner than could conceivably lead to the apparent differences. It is possible however, that the tissue used in Chapter 3 (which was the same for all the examined Methods) was not representative. This experiment has demonstrated that there are individual variations in enzyme activities which do not reflect the trends described in Table 4.3. For example, the values of calpain I and II reported from animals 1 and 8 do not reflect the reported 1:1 ratio. Thus, additional samples need to be examined to establish the natural variations in muscle enzymes in sheep.

4.4.2 Temperature, tenderness and pH

The temperature and pH profiles over the 24 hour post-mortem chilling period conform to the predicted trends for ovine muscle over this conditioning period. The temperature decline is directed by storage conditions. pH decline is indirectly influenced by temperature via its regulation of post-mortem glycolysis and the accumulation of lactic acid. Both temperature decline and pH influence enzyme activities (Geesink 1993).

4.4.3 Correlation of enzymes to tenderness

There is a poor correlation between muscle protease activity and meat tenderness. Figure 4.3 shows that there is a slight positive correlation between protease level and toughness (Calpain I vs Kgf = 0.097, Calpain II vs Kgf = 0.065). This contradicts other work where high proteolytic enzyme activity has been associated with tender meat. It is important to point out that the r^2 values for the correlation of calpain I/II to toughness are very low (<0.005). This indicates that the illustrated correlation lines are poor representations of the widely scattered data points. Calpastatin shows a positive correlation with toughness (Calpastatin vs Kgf = 0.269). This is expected since it is responsible for the inhibition of the calpains and thus a reduction in proteolytic tenderisation. While the r^2 value of this correlation is greater (0.07), it is not significant. This failure to demonstrate an association between calpain activity and tenderness illustrates that it is important to maximise the number of samples when analysing trends.

4.5 Summary

This chapter has revealed a number of inconsistencies with respect to the quantification of calpains and calpastatin. Firstly, there is a significant irregularity in enzyme levels between individuals. Thought must be given to maximising sample numbers and reducing this variation. Another factor is the differences in absolute enzyme activity yielded between these experiments and those reported by other workers. It is important to be confident that results obtained by one technique are comparable with others experimental results are to be compared. I am not certain that the protocols described above were performed in accordance with the work of other researchers. However, this methodology does give consistent separation of the proteolytic system and allows reproducible quantification of the activity. In order to maintain the continuity of this thesis, the protocols described above will be used in following chapters.

Chapter 5:

Characteristics of calpain activities over time in post-mortem ovine muscle.

5.1 Introduction.

It is generally accepted that the rate of meat tenderisation is dependent upon the activity of the calpain proteases and their inhibitor calpastatin (Dransfield 1993; Koohmaraie 1994; Wheeler *et al.*, 1991a). Tenderisation begins when calpain I becomes active and attacks the Z disk component of the muscle fibre (Dransfield 1994). As a consequence, the toughening that takes place during rigor development is reduced post-rigor. Approximately 50% of tenderisation occurs in the 24 hours immediately post-slaughter although this depends upon the muscle pH and temperature (Dransfield 1992b). After 24 hours the rate of tenderisation tapers off and reaches a plateau (Dransfield 1994). Under normal post-mortem storage conditions the ultimate tenderness of a lamb carcass is reached within 7-14 days. These changes in tenderisation correlate positively with declines in carcass temperature, pH and ATP (Whipple *et al.*, 1990; Koohmaraie *et al.*, 1987) and with increases in free Ca^{2+} concentrations (Etherington 1991). These variables are interconnected, but one important relationship is between the drop in ATP and the rise in intracellular Ca^{2+}. Under *in vivo* conditions Ca^{2+} pumps are found in the sarcoplasmic reticulum (SR) and maintain homeostatic intracellular Ca^{2+} concentrations. However, after slaughter, and as a result of the depletion of ATP stores, there is cessation in their activity and intracellular Ca^{2+} rises. As a result, the activity of calpain I increases rapidly after death. This rise takes place within the 6-10 hour period post mortem and represents a significant increase over the basal calpain activities. The extent of elevation is dependent upon pH, temperature and the stress the animal is subjected to prior to slaughter. Maximum calpain I activity is realized after about 10 hours and then declines rapidly. Dransfield (1993) has calculated that this decline is such that only 18% of the initial levels are present at 48 hours post-slaughter. This rapid loss of activity, coupled with increasing meat tenderness, indicates that calpain I is an important protease involved in the conversion of muscle into meat (Etherington 1991).

Calpain II activity begins to increase after 16 hours, reaches a maximum by 20 hours, and then slowly declines over several days. The absolute maximum activity attained by calpain II is significantly less than that for calpain I (Dransfield 1994), although the exact activities vary from animal to animal, and between species. This delayed increase and reduced activity compared to calpain I arises for three reasons: 1) Intracellular free Ca^{2+} concentrations are sufficient to activate all the calpain I protease but will only activate 30% of the total calpain II. 2) Calpain II is maximally active at pH 7.5 and would therefore display only a fraction of the normal post-mortem storage pH of 5.5. 3) Calpain II has been found to possess maximum proteolytic activity at 20°C whilst most tenderisation occurs at the storage temperature of 2-4°C (Koohmaraie 1988).

Thus tenderisation commences with the rise in activated calpain I and toughness subsequently declines rapidly reaching about 50-60% of the initial toughness after 24 hours. Tenderisation after 24 hours is caused by residual calpain I and the lesser calpain II activities. The slight increase in tenderness after 6 days is due to calpain II only.

The cessation of calpain I and II activities is a result of their autolysis (McClelland *et al.*, 1989; Mellŏni *et al.*, 1984). Under experimental assay conditions (5mM Ca^{2+} concentration) after 60 minutes the activity of calpain I is only 17% as high as it was before autolysis began. Calpain II is only 6% (Edmunds *et al.*, 1991). This suggests that the process of deactivation takes place at an exponential rate (Koohmaraie 1987), and work by (Edmunds *et al.*, 1991) has indicated that autolysis of calpain II is an intramolecular process whereas calpain I autolysis is intermolecular.

The purpose of this chapter was to examine the effect of time (post-slaughter) on calpain activities *in vitro*. Calpain activity was measured at 6 time intervals post-mortem. Following a survey of current literature it was decided to group these times so that the early post-mortem period (up to 48 hours) were well defined.

5.2 Materials and Methods.

It was decided to use the DEAE Sephacel bulk elution technique (Method 3 analyzed in Chapter 3) on the basis of its speed of separation, and the limited drain on resources. Calpastatin was not measured.

5.2.1 Calpain extraction.

These experiments employed a modified version of the process described by Thomson *et al.*, (1991). A whole *longissimus dorsi* muscle was removed from a Coopworth lamb immediately after slaughter, and maintained at 4°C for the duration of this experiment. The effect of 0, 6, 24, 48, 72 and 144 hours post-slaughter storage on muscle proteases was determined. At each of these times a 5gm subsample of muscle was trimmed of visible fat and connective tissue, minced with a scalpel and scissors and then homogenised in 30ml extraction buffer (50mM TRIS-Hcl, 10mM ß-MCE, 10mM EDTA, and 0.2% Triton X-100, pH 7.5). The homogenate was spun at 32000rpm for 60 min at 4°C and the supernatant decanted. The conductivity of supernatant was adjusted to 20mM using distilled deionized water then loaded onto a 1 x 20cm econo-column containing 5ml DEAE Sephacel (pre-washed with equilibration buffer). Once the sample had drained through the column, the column was washed with 30ml equilibration buffer (40mM TRIS-HCl, 0.5mM EDTA, 10mM ß-MCE, pH 7.5). Subsequently the column was eluted with 6 column volumes of 100mM NaCl in equilibration buffer, 6 column volumes of 200mM NaCl in equilibration buffer and 6 column volumes of 400mM NaCl in equilibration buffer. A total of eighteen 5ml fractions were collected.

5.2.2 Measurements of activities

Each fraction was assayed for calpain activity. 0.2ml from fractions 1-12 was incubated with 0.2ml Ca^{2+} casein (10mM TRIS-HCl, 5mM $CaCl_2$, 1mM NaN_3, 5mg/ml Casein, 10mM ß-MCE, pH 7.5) for 60 minutes at 25°C. The reaction was stopped by adding 0.4ml ice cold 5% (TCA). The assay mixture was centrifuged at 4000rpm for 15 minutes to remove the precipitate and the absorbance of the supernatant was measured at 278nm. This measurement reflects the amount of protein hydrolysed by Ca^{2+} dependent proteolysis under these standard assay conditions. The Ca^{2+} independent hydrolysed protein was determined in the same manner using EGTA containing casein (10mM TRIS-HCl, 10mM EGTA, 1mM NaN_3, 5mg/ml Casein, 10mM ß-MCE, pH 7.5). Each fraction was assayed in duplicate with each assay media. For fractions 13-18, 0.1ml of the fraction was incubated with each of casein medium and the Ca^{2+} dependent activity of each fraction was multiplied by 2 to produce results comparable with those for fractions 1-12. Calculation of calpain I and II activities (units/gm tissue) were performed using Equations 1 and 2 in Chapter 3.

5.3 Results.

5.3.1 Experiment 1

Table 5.1 and Figure 5.1 show activities of skeletal muscle calpain I and II decline with time.

	0 Hours		*6 Hours*		*24 Hours*	
	Calp I	Calp II	Calp I	Calp II	Calp I	Calp II
No. Fractions	1	2	3	2	2	2
Fraction Size (ml)	5	5	5	5	5	5
Sample Size (gm)	5	5	5	5	5	5
Average Activity (per ml, 278nm)	1.76	1.59	0.42	1.48	0.29	1.64
Units/gm	1.76	3.18	1.26	2.96	0.58	3.28
	48 Hours		*72 Hours*		*144 Hours*	
	Calp I	Calp II	Calp I	Calp II	Calp I	Calp II
No. Fractions	1	2	1	2	1	2
Fraction Size (ml)	5	5	5	5	5	5
Sample Size (gm)	5	5	5	5	5	5
Average Activity (per ml, 278nm)	0.10	1.70	0.11	1.21	0.12	0.84
Units/gm	0.10	3.40	0.11	2.42	0.12	1.68

Table 5.1 Activities of calpains I and II (units/gm tissue) from post-mortem muscle at six sampling periods. Also included are the variables required for their determination.

It would seem from Figure 5.1 that calpain I levels drop more rapidly than calpain II. This observation agrees with that reported by Koohmaraie *et al.*, (1987). After 48 hours calpain I activity is only 6% of the original whereas calpain II still retains over 53% of its initial activity at 14 hours.

Time (Hrs post-mortem)	*0*	*6*	*24*	*48*	*72*	*144*
Calpain I	1.76	1.26	0.58	0.10	0.11	0.12
Percent of Time 0	100	72	33	6	6	7
Calpain II	3.18	1.26	3.28	3.40	2.42	1.68
Percent of Time 0	100	93	103	107	76	53

Table 5.2 Activity (units/gm) of calpain I and II in post-mortem muscle over 144hr period.

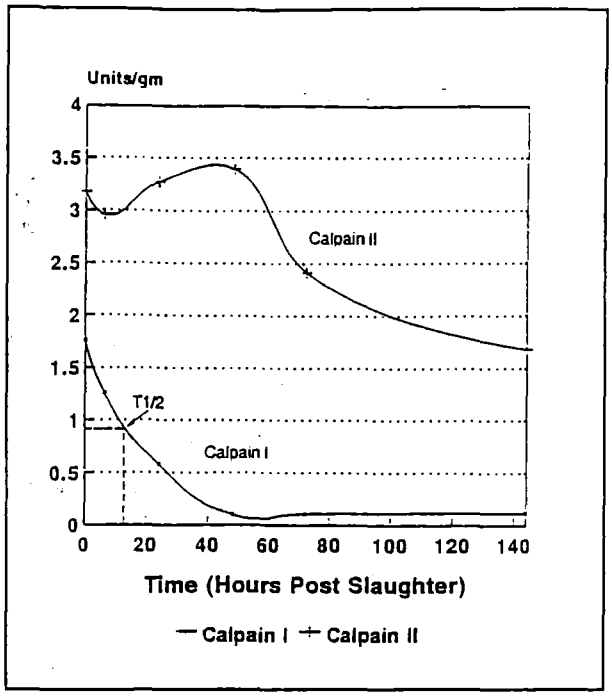

Figure 5.1 Calpain activities decile with time. Calpain I and II levels in LD muscle stored at 4°C. Calpains were extracted using a DEAE bulk elution method, and assayed following standard assay procedure. The graph includes the hypothetical position of the T½ for calpain I.

In concordance with the results of other workers (Edmunds 1991; Dransfield *et al.*, 1992b) the rate of calpain I decline appears to be exponential. The time frame of the decay is, however, faster than has been reported else where.

5.3.1.1 Discussion

Table 5.2 indicates that 50% of calpain I activity is lost between 6 and 24 hours post-mortem. The extrapolated line in Figure 5.1 approximates the T½ of calpain I to be 15 hours. This differs markedly from Koohmaraie *et al.*, (1987) who observed that a 50% drop in calpain I only occurred after 24 hours. Breed and muscle differences in the levels and activities of the calpains have been previously observed (Ouali *et al.*, 1990). The muscles used in Koohmaraies' work were LD taken from cattle, and this may explain the observed contrast. The decline in calpain II is more gradual than calpain I. The T½ of calpain II is estimated at 7-10 days which is sooner than that reported by Koohmaraie *et al.*, (1987). Koohmaraie estimated that by 14 days, 80.2% of the original calpain II was present which equates to a T½ of more than 1 month.

Interestingly, the activity of calpain II escalates between 6 and 48 hours post-mortem, after which the activity tapers off in a near linear trend. The increase in calpain II agrees with other work (Dransfield 1994) and possibly results from increased availability of Ca^{2+}. No drop was observed in calpain II in the initial 24 hour period in agreement with Koohmaraie *et al.*, (1990a). A relatively rapid drop to 76% of the original activity was seen between 48 and 72 hours after which time the decline levelled off.

5.3.2 Experiment 2

Another experiment was conducted to assess the changes in calpain activity over the initial 5 hour post-slaughter period. The experiment was performed as previously described in the Materials and Methods section at 1, 2, 3, 4, and 5 hours post-mortem. Figure 5.2 displays the results graphically and tables 5.3 and 5.4 the individual data.

	1 Hour		*2 Hours*		*3 Hours*	
	Calp I	Calp II	Calp I	Calp II	Calp I	Calp II
Units/gm	1.54	3.28	1.25	3.32	0.90	3.50
	4 Hours		*5 Hours*			
	Calp I	Calp II	Calp I	Calp II		
Units/gm	0.71	3.28	0.76	3.18		

Table 5.3 Activities (units/gm) of calpains I and II from post-mortem muscle at five time intervals post-slaughter. Calpains were extracted following a DEAE based bulk elution technique, and assayed under standard conditions.

Confirming our earlier work there was a significant drop in the activity of calpain I in the initial 5 hour period post-mortem. There was little measurable changes in calpain II activity.

5.3.2.1 Discussion

In Figure 5.1 the T½ for calpain I was approximately 15 hours. In the previous experiment the T½ is hypothesised to be between 3 and 4 hours post-mortem. This difference is probably due to sample variation since the storage and measurement conditions were the same in both experiments.

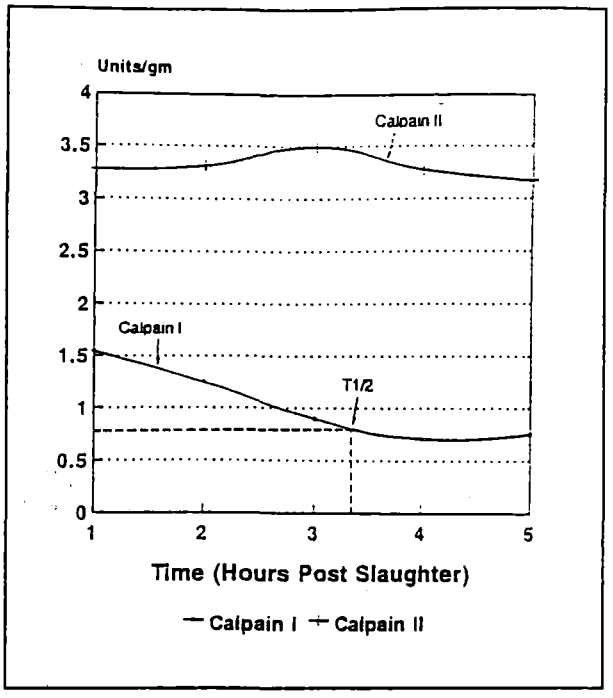

Figure 5.2 Variation in calpain I and II activities in the LD muscle over five hours post-mortem. Muscle was stored at 4°C for the duration of the experiment. Graph includes the hypothetical position of the T½ for calpain I.

There are two possible explanations for the results. Firstly, it may be that sample 2 had higher intracellular Ca^{2+} levels which would accelerate autolysis of the calpain I protease. Usually however, animals maintain relatively stable homeostatic levels of ions such as Ca^{2+}, and therefore this is probably an unlikely explanation. Secondly, the slaughter conditions may have caused the first animal to suffer more stress which would raise its post-slaughter pH. Calpain I has a pH optimum of approximately 7, and higher pH possibly retards its activity. This could then appear as a reduced activity *in vivo* and a lower rate of self proteolysis.

Time (Hrs post-mortem)	*1*	*2*	*3*	*4*	*5*
Calpain I	1.54	1.25	0.90	0.71	0.76
Percent of Time 0	100	81	58	46	49
Calpain II	3.28	3.32	3.50	3.28	3.18
Percent of Time 0	100	101	107	100	97

Table 5.4 Tabulated results showing units/gm of calpain I and II over time. Percentage changes (when compared to time 0) is also displayed.

5.4 Conclusion and Discussion

The bulk elution (Method 3 in Chapter 3) appears to have been successful in extracting the calpain proteases. The reliability of this protocol was assessed in this experiment (data not shown) by simultaneously performing three extractions on samples of meat taken from the same muscle. Calpain I and calpain II were separated, and the enzyme yields from each column were within 5% confidence interval.

As noted in the results section, there is a relatively rapid drop in both calpain I and II in these experiments compared to the results presented by other researchers. It is difficult to be certain of the reasons for the differences but it may be due to variations in the experimental protocol or in the tissue samples analyzed. In this work the same muscle from different sources was compared. Different T½'s were observed despite a uniform experimental protocol. This evidence alone supports the hypothesis that differences in samples can lead to differences in the characteristics of the proteases.

The observations on the time dependent decline of calpain activity in these experiments agree with those made by other workers (Koohmaraie *et al.*, 1987; Dransfield 1992b). Total activity of calpain I fell rapidly post-mortem, whereas calpain II levels remained relatively stable before gradually declining. Post-mortem muscle pH fell from 6.7 to 5.5 due to the conversion of anaerobic respiration converting glycogen into lactic acid. This in turn leads to the formation of cross bridge links between the muscle thick (myosin) and thin (actin) filaments and the generation of tough meat (Etherington 1991). However, rigor development is temporary and the muscle enters into a tenderisation stage once certain criteria (such as rise in Ca^{2+}) are met. The conditioning stage represents the conversion of muscle to meat. The rapid drop in calpain I, seen in Figure 5.1, reflects its action in the conditioning process. As the ATP content in the muscle tissue declines, the activity of the Ca^{2+} pumps found in the sarcoplasmic reticulum (SR) slows to a halt. This, coupled with proteolytic attack of the SR, leads in an inability of the Ca^{2+} pump to maintain homeostatic Ca^{2+} levels and a significant rise in Ca^{2+} becomes apparent. Soon after death the Ca^{2+} ion concentration is approximately 10^{-7}M in the sarcoplasmic reticulum, which is too low to activate the calpains and their inhibitor. However, after the pH of the muscle tissue has dropped to about 6.1 the Ca^{2+} concentration rises to 10^{-4}M which is enough to maximally activate some of the muscle calpains (Dransfield 1992). Calpain I is activated and proteolytically attacks the Z disc of the muscle myofibril. This leads to a fragmentation of the fibrillar structure and an overall

tenderisation of the meat product. Koohmaraie (1988) has determined despite the presence of a substrate Z disc, that the calpains also execute self destructive autolysis which leads to 100% degradation of the calpains. Thus the actual activity of calpain I in the conditioning process is mirrored in self destructive autolysis and its overall decline. Thus just as calpain I degrades the Z-disc it also degrades itself.

Calpain II appears to maintain a relatively high activity (units/gm) during the first 48 hours post-mortem. The fact that there is little change in the units/gm during this time suggests that calpain II is not undergoing autolysis and is not especially active in the tenderisation process. After 48 hours, Figure 5.1 showed a decline in the amount of calpain II extracted from meat. This decline is caused by the protease undergoing autolysis. It is likely that there are two reasons for this delayed activity. Firstly the free Ca^{2+} reaches 100% after approximately 24 hours (Etherington 1991) and this high level of Ca^{2+} is demanded for the activation and operation of calpain II. However, because calpain I is still present in sufficient concentrations to sequester the free Ca^{2+}, calpain II remains relatively inactive. It will remain inactive until levels of Ca^{2+} are 100% and until calpain I has undergone autolysis and released the Ca^{2+} it was sequestering. Once calpain II becomes active it participates in the conditioning process by degrading the Z-disc component and also undergoes intermolecular autolysis (Edmunds *et al.*, 1991) which is responsible for its gradual decline. This has been shown in Figure 5.2.

The increase in calpain II activity at 24 and 48 hours is puzzling. Two suggestions may explain this. Firstly, it may be that more calpain II is being extracted from the muscle tissue, and secondly, the calpain II being extracted may be more active at these intermediate times (24-48 hrs) than when fresh. There have been a number of papers suggesting that for reasons of enzyme activation, calpain II binds to components (protein or lipid) of the plasma membrane (Pontremoli *et al.*, 1985; Salamino *et al.*, 1993). During the extraction process a centrifugation step is used to remove plasma membrane lipid and it is possible that calpain II bound to the membrane is also discarded. As the muscle tissue ages, sources of energy (eg., ATP) responsible for maintaining the cellular integrity dissipate and a loss of cell structure takes place. This loss of structure is probably displayed as plasma membrane fragmentation and the liberation of integral components. Therefore, it may be that as the tissue ages calpain II is freed from the membrane and not lost during the separation stage. However, this explanation does not seem very likely since Triton X-100 (a biological detergent) is included in the extraction buffer and should be able to completely fragment the plasma membranes during tissue preparation. A reason why calpain II might be more active at

intermediate times can be explained by the theory that calpain II binds to a membrane bound calpain activator protein (Salamino *et al.*, 1993). This protein promotes the autoproteolysis of the proteinase at Ca^{2+} levels near physiological levels. In fact, the activator protein decreases the Ca^{2+} concentration required for activation of calpain II by approximately 100 times. Thus it seems likely that as free Ca^{2+} levels rise (post-mortem) there is a mechanism that promotes the conversion of inactive procalpain II to the active isoform. This would reflect as an increased calpain II activity yielded from the tissue.

The gradual decline in calpain II taking place in the later times seems likely to be due to the intramolecular process of autolysis that Edmunds *et al.*, (1991) has previously described. Again, it should be pointed out that the autolysis of the calpain enzyme requires its activation, and the observation of a rapid decline also reflects the enzymes proteolytic action on cellular components.

Although calpastatin levels were not determined in this work, a number of other experimental papers have established the nature of its behaviour in muscle tissue post mortem. Whipple *et al.*, (1990) reported a 50% decrease in calpastatin activity (from fresh muscle) between 0 and 24 hours post-mortem. She proposes that the reason for this decline is that activated calpain hydrolyses the inhibitor molecule. However, calpastatin can be degraded by several thiol proteinases found in muscle post-mortem. Dransfield (1993) determined that calpastatin decline is rapid, and has calculated that the decline leaves only 30% of the original activity at 24 hours. He agrees that the decrease is probably due to calpain mediated hydrolysis. Koohmaraie *et al.*, (1987) concluded that calpastatin was very susceptible to degradation post-mortem, and that after 24 hours only 20.7% of the original inhibitor activity was present. By 6 days the activity was virtually eliminated.

5.5 Summary

This chapter has explored the changes in the activity of calpain proteases post-mortem. The conclusion is that calpain I is the most responsible for the tenderisation of meat, and that most of this tenderisation (and concomitant calpain I decline) takes place early in the conditioning process. Calpain II is probably involved only in residual tenderisation and does not decline rapidly post-mortem. Although not measured, calpastatin is reported to drop very rapidly probably as a result of calpain I hydrolysis. These observations have important implications for understanding the mechanistic model for tenderisation. This work emphasises that when quantifying components of the calpain system post-mortem careful consideration of the time effect must be made. There have

been various attempts to stabilise the system components including freezing and incorporation of protease inhibitors but a reliable protocol for storage has remained elusive.

Future work in this area would benefit from experiments taking into account all the relevant factors mentioned here. These would include quantification of the calpain I, calpain II and calpastatin, and measurement of tenderness, pH, Ca^{2+}, ATP levels pre- and post-slaughter.

Chapter 6:

Differences in the activity of calpains and inhibitor calpastatin in two lines of sheep differing in their glucose tolerance.

6.1 Introduction.

At Lincoln two lines of Coopworth sheep have been bred which exhibit differences in glucose tolerance. These animals were derived from an original flock which was screened for its tolerance to glucose using the glucose tolerance test (GTT). The GTT consists of a rapid infusion of glucose into the venous blood system which induces an insulin response. The then causes a decline in plasma glucose. The time (in minutes) for half the glucose load to be removed from circulation is referred to as T½, and is a measure of an animals tolerance to plasma glucose. In early 1987 the T½ of 350 Coopworth ram animals were determined. From this flock seven ram animals displaying the lowest T½ (fast glucose clearance) were termed Fast sires, and the seven with the highest T½ (slow glucose clearance) were called Slow sires. Each ram was mated with 25 mixed age Coopworth ewes allocated at random. Rams from the resulting progeny (generation 1) were screened as before and then mated with a new flock of ewes. This process was repeated a second and third time to produce second and third generation progeny. By early 1990 these matings had given rise to 2 flocks of sheep with significant differences in their tolerance to glucose. Sue Francis (1990) wrote a comprehensive PhD thesis which examined the physiological differences between the two lines of sheep. Her experiments concluded that the Slow line animals had a significantly ($p<0.05$) slower rate of glucose clearance than the Fast line. The resting plasma glucose was significantly ($p<0.05$) higher in the Slow animals than Fast animals. Resting insulin levels did not significantly differ. However, following a glucose challenge the level of plasma insulin was significantly higher ($p<0.05$) in the Fast than Slow sheep. Body composition differences were apparent in an examination of subcutaneous fat depth over the 12th rib at the GR position. Fast animals displayed significantly greater depths of subcutaneous fat than the Slow. Concordantly, the percentage of fat in the whole carcass was significantly ($p<0.05$) less in the Slow than the Fast line. The same trend was observed for the depth of the *longissimus dorsi* (LD) muscle. The Fast line of sheep had significantly ($p<0.05$) greater LD depth than the Slow. Unexpectedly, the total carcass protein was significantly greater in the Slow than in the Fast sheep. Francis (1990) pointed out however, that the muscle depth measurements made at one site on one

muscle may not reflect the pattern of the whole muscle mass. Plasma urea, which reflects protein degradation, was lower in the Slow than the Fast line. Another muscle parameter of interest is pH, and other researchers (Shorthose 1970) have observed a positive correlation between T½ and muscle pH. This would suggest that Fast animals may have a higher muscle pH than Slow. However, in her work, Francis (1990), observed there were no significant pH differences between the two lines of animals. Table 6.1 summarises these observations.

	Slow (Long T½)	Fast (Short T½)
Glucose clearance rate	Lesser	Greater
Resting plasma glucose	Greater	Lesser
Resting plasma insulin	No difference	No difference
Glucose challenge plasma insulin	Lesser	Greater
Subcutaneous fat	Lesser	Greater
% Fat (whole body)	Lesser	Greater
Muscle depth (*longissimus dorsi*)	Lesser	Greater
% Protein (whole body)	Greater	Lesser
Plasma Urea	Lesser	Greater
Muscle pH	No difference	No difference

Table 6.1 Table showing trend of differences between Fast and Slow glucose clearance Coopworth sheep (adapted from Francis 1990).

6.1.1 Physiological effects of insulin

Insulin is secreted by the pancreas in response to plasma glucose levels and consequently is concerned with maintaining homeostatic plasma glucose levels. Three primary anabolic function of insulin are the sequestering of glucose, lipogenesis and protein deposition in muscle (Willis 1990).

Insulin inhibits gluconeogensis and increases the peripheral uptake of glucose by stimulating the translocation of glucose transporter molecules (GLUT's) to the cell surface (Kahn 1985; Kasanicki *et al.*, 1990). In addition, insulin inhibits hepatic glucose output, encourages glycogen synthesis and alters the availability of glucose precursors to the liver (Brockman 1986).
The lipogenic effects of insulin results from its stimulation of lipoprotein lipase (LPL), and its inhibition of hormone sensitive tissue lipase. Anti-lipolytic effects include reduced glycerol release and the reduced availability of glucogenic amino acids to the liver (Brockman 1986).

Muscle tissue represents a major protein store. Insulin increases the rate of amino acid uptake into muscle, raises the concentration of RNA and promotes the aggregation of polysomes (Charles 1986). These actions serve to promote protein synthesis, but it is thought that this alone does not account for the observed increase in protein levels. It has been suggested that insulin may have a depressive effect on muscle protein degradation which leads to a net increase in protein deposition (Doherty 1992; Francis 1990). However, there is some disagreement since an increase in total protein may arise from either a decrease in protein degradation and/or an increase in protein synthesis. Thus the appropriate modification of either of these parameters could produce a net increase in protein content. Indeed, some researchers (Brockman *et al.*, 1986; Weeks 1986) have concluded that insulin promotes protein synthesis and depresses degradation. Other workers have observed a decline in protein synthesis and a greater decline in degradation (Charles 1986; Oddy *et al.*, 1987). Some workers have concluded that there is an increase in both protein degradation and synthesis, with the former increase being out weighed by the latter (Crompton *et al.*, 1987).

Thus, the manner in which the balance of protein degradation and synthesis is disrupted (leading to a net increase in protein levels) is unclear, but depression of proteolysis is conceivably responsible for this change. The calpain proteolytic system is common to all animal cells and may be responsible for a significant portion of the protein degradation witnessed in muscle tissue. It has been stressed previously that a better understanding of the calpains and their inhibitor should be developed in order to fully appreciate their potential role in nutrient partitioning (Bickerstaffe 1993). This trial was performed to determine if previously reported physiological differences arising from differences in glucose tolerance were correlated with changes in the calpain protease system and, more specifically, to investigate if the observed differences in protein content (Table 6.1) were related to differences in the activity of components of the calpain system. Previous experiments have shown that the Slow animals have a greater percentage body protein and lower plasma urea. It is possible that this may be the result of a depressed proteolytic system.

6.2 Methods.

This trial was designed to assess the effects of glucose tolerance on the calpain proteases and their inhibitor calpastatin. Other parameters such as body composition and insulin levels were also measured. Calpain and calpastatin activities were determined by ion exchange chromatography as outlined below. pH was measured using a Hanna HI9025C pH meter with standard temperature probe, and an Orion 8163SC combination spear tip pH probe. Shear force of 40mm chops from 1 hour LD muscles was determined using a Tenderometer (B.R.Homersham Ltd) and following MRINZ specifications.

6.2.1 Animals

The animals used in this experiment were offspring from previously established High and Low glucose tolerance sheep lines. The average birth date of the animals was September 1st 1992. Variation around this time was 1 week. Animals within the High and Low lines were balanced for sex. 64 animals were maintained for the duration of the trial at the Templeton Research Station, Canterbury. Animals were kept on pasture and weaned according to normal practice. There was no restrictions on feed consumption at any time. Slaughter of the animals took place in the last week of March 1993. For convenience, the animals were randomly allocated to one of three groups. The first group was slaughtered on Monday, the second Wednesday and the last on Friday.

6.2.2 Calpain system separation and assay

Calpains were prepared from 40gm sample of the *longissimus dorsi* muscle taken from 20 ram animals (10 High, 10 Low), 20 minutes after death. A 5gm subsample of the muscle was trimmed of visible fat and connective tissue before minced with scissors and a scalpel. The sample was then homogenised in 5 vols (25ml), of cold extraction buffer (100mM TRIS-HCl, 10mM ß-MCE, 10mM EDTA, pH 8.3). Homogenisation took place in a 250ml Waring Blender cup using a Waring Blender set on high speed. Three 30 second homogenisations were separated by cooling periods of 30 seconds. The homogenate was centrifuged at 15,000 rpm for 1 hour at 4°C, the supernatant decanted off and filtered through a washed glass wool/cheese cloth sandwich. The pH of the filtered extract was adjusted to 7.5 with 1M acetic acid and dialysed for 18 hours against dialysis buffer (40mM TRIS-HCl, 3mM EDTA, 10mM ß-MCE, pH 7.5) at 4°C. The extract was filtered through a washed, moist glass wool/cheese cloth sandwich and then loaded onto a DEAE-sephacel column (1.5 x 20cm) which had been equilibrated with elution buffer (40mM TRIS-HCL, 0.5mM EDTA, 10mM ß-MCE, pH 7.5). The column was washed with 250ml elution buffer and the bound proteins eluted by a continuous gradient of NaCl in elution buffer from 25mM to 350mM (120ml of each).

Calpastatin was extracted from the sample following the above centrifugation step. A 1ml subsample of the extract was boiled at 95°C for 15min before being spun at 11000 rpm for 5min. The resulting supernatant was assayed for calpastatin.

The activities of calpain I and II were determined using casein as a substrate. Briefly, 0.5ml of fractions 1-55 and 0.25ml of fractions 56-80 were combined with appropriate volumes of Ca^{2+} and

EGTA casein (10mM TRIS-HCl, 1mM NaN_3, 10mM ß-Mercaptoethanol, 5mg/ml Casein, 5mM $CaCl_2$ or 10mM EGTA, pH 7.5) to bring the total volume up to 1.0ml. The mixture was incubated at 25°C for 1 hour and the reaction stopped by the addition of 1.0ml ice cold 5% TCA. Assay tubes were spun at 4000rpm for 15 min and the soluble digestion products in the supernatant measured spectrophotomerically at 278nm. One unit of calpain activity is defined as the amount of enzyme catalysing an increase of 1 absorbance unit at 278nm in 60min at 25°C.

Calpastatin was assayed by incubating a 250µl subsample of the soluble calpastatin fraction with a volume of calpain II possessing an activity of 0.25-0.30 units. Loss of activity in these tubes, when compared with control (calpain only), represents calpastatin activity.

6.2.3 Glucose tolerance test

Glucose tolerance tests were performed at 20 weeks. For a GTT a single shot of glucose (0.3mg/kg liveweight) in 50% saline solution was injected into the jugular vein using a butterfly-winged infusion set. Blood samples were taken into evacuated blood collecting tubes at -10, 0, 10, 20, 40, 60 and 90 minutes relative to the glucose challenge. The blood was centrifuged for 20 minutes at 1000g, and plasma stored at -20°C until analyzed for glucose and insulin.

6.2.4 Carcass measurements

At 26 weeks measurement of hot carcass weight and subcutaneous fat over the 12th rib at the GR position were made at Templeton. One half of the carcass was retained and frozen for subsequent mincing and carcass analysis of total fat and protein.

After slaughter, loins from both sides of the carcass were cut into 40mm chops to yield 8 chops per animal. The chops were stored at 1°C and at 0, 4, 8, 10, 12, 22, 30, 40 and 627 hours post-slaughter pH and tenderness were measured using a MIRINZ tenderometer and following standard procedures (Quality Assurance for Tenderness Revision of MIRINZ Rm 126 technical report, May 1991 MIRINZ 872, ISSN 0465-4390).

6.2.5 Statistical analysis

Data was tabulated in Quattro Pro spreadsheets before being analyzed using Minitab Statistical Software. Data was analyzed using ANOVA and Students T Test.

6.3 Results.

6.3.1 Calpain system quantification

The protocols described have been successful in achieving the separation and quantification of calpain I and II. Figure 6.1 shows two clearly defined peaks of protease activity. Table 6.2 presents the units/gram of calpains I and II extracted from the tissues, and Table 6.3 the units per gram calpastatin.

A total of 20 animals were examined. There was some irregularity in the location of each peak, but this is probably due to differences in individual fraction collector characteristics rather than separation properties.

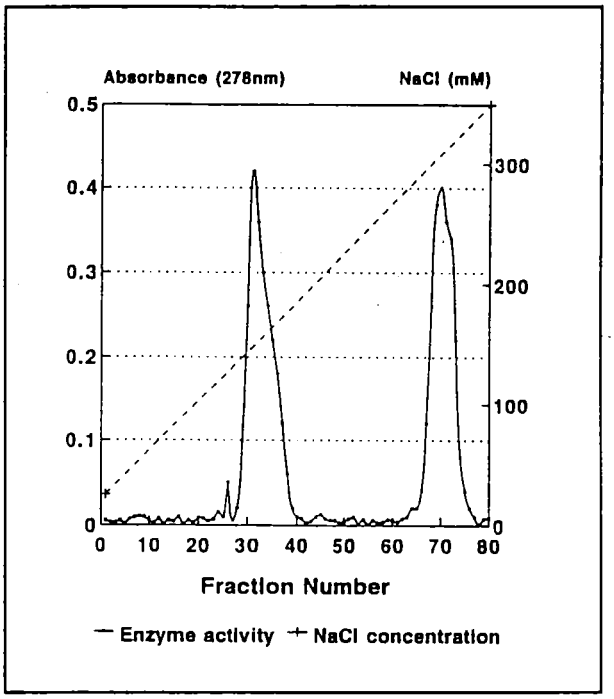

Figure 6.1 Graph of protease activity taken from the longissimus dorsi from one Fast T½ animal. Fractions were obtained from a 20 x 1cm DEAE Sephacel column using a linear NaCl gradient (25-350mM), 80 3ml fractions were collected. Assays were performed following a standard assay protocol.

Table 6.2 presents the units per gram calpain I and II in each sample. Calculation of units per gram are as described in chapter 3.

Animal Number	T½	Calpain I (Units/gm)	Calpain II (Units/gm)
1	Slow	1.12	0.72
2	Slow	0.52	1.24
3	Slow	1.07	0.52
4	Slow	0.99	0.30
5	Slow	0.72	0.40
6	Slow	0.86	1.00
7	Slow	0.69	0.88
8	Slow	0.57	0.60
9	Slow	0.43	0.88
10	Slow	0.32	0.24
Average	Slow	0.73 (±0.3)	0.68 (±0.3)
11	Fast	1.26	1.12
12	Fast	0.55	2.42
13	Fast	1.03	0.78
14	Fast	0.51	0.50
15	Fast	0.83	1.06
16	Fast	1.12	1.38
17	Fast	1.46	0.94
18	Fast	1.42	0.86
19	Fast	0.91	1.18
20	Fast	1.52	0.58
Average	Fast	1.06 (±0.4)	1.08 (±0.5)

Table 6.2 Units/gm yield of calpain I and II from 20 Coopworth rams differing in glucose clearance T½.

The activities of calpain I and II show considerable variation between samples from the same line and between lines of animals. In addition, calpain units/gm are less than those obtained in previous chapters. Calpain I and calpain II yields from the Fast are significantly ($p<0.05$) higher from the Fast than Slow line.

Animal	Line	Calpastatin (Units/gm)	Animal	Line	Calpastatin (Units/gm)
1	Slow	0.45	11	Fast	0.21
2	Slow	0.14	12	Fast	0.15
3	Slow	0.37	13	Fast	0.20
4	Slow	0.07	14	Fast	0.07
5	Slow	0.10	15	Fast	0.10
6	Slow	0.18	16	Fast	0.09
7	Slow	0.37	17	Fast	0.03
8	Slow	0.30	18	Fast	0.03
9	Slow	0.27	19	Fast	0.09
10	Slow	0.04	20	Fast	0.12
Average	Slow	0.23 (±0.1)	Average	Fast	0.11 (±0.06)

Table 6.3 U/gm Calpastatin extracted from Coopworth Rams with different glucose clearance.

Table 6.3 shows that the activities are significantly ($p<0.05$) higher in the Slow than the Fast line.

6.3.2 Glucose tolerance test

As expected, there are significant differences between the two lines of sheep to their glucose tolerance. Changes in plasma glucose, with respect to time, after a single injection of glucose are shown in Figure 6.2. The insulin response to the glucose infusion is shown in Figure 6.3.

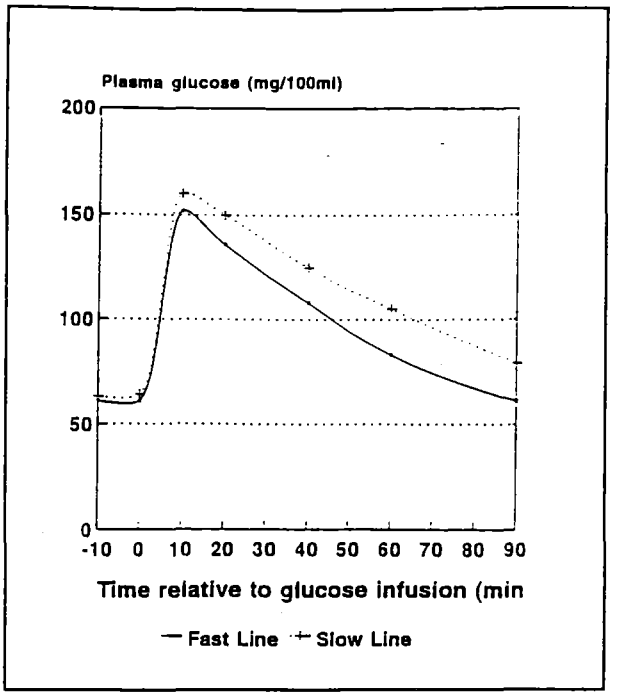

Figure 6.2 Changes in plasma glucose concentration following a glucose challenge of 3mg/kg bodyweight. Graph shows differences between High and Low glucose tolerance sheep lines.

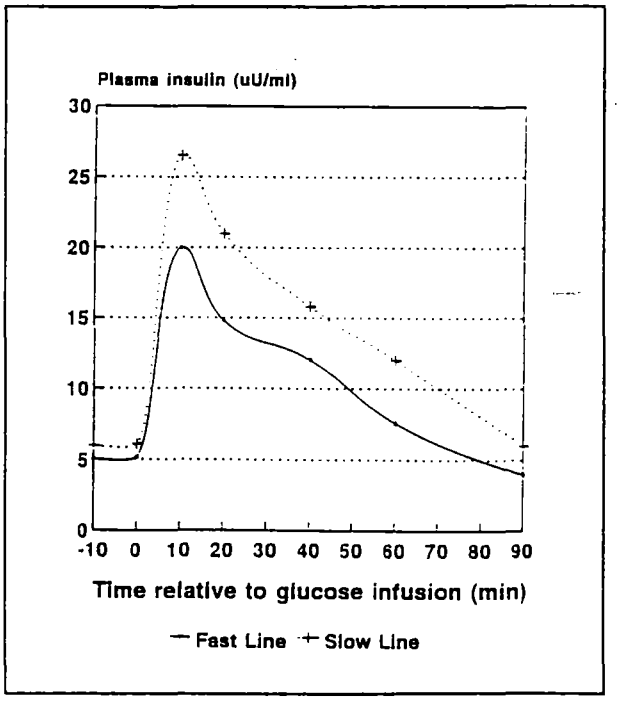

Figure 6.3 Changes in plasma insulin concentration against time following a glucose challenge of 3mg/kg bodyweight. The graph shows differences between the High and Low glucose tolerance sheep lines.

Table 6.4 presents the area under each curve for the glucose and insulin response curves following glucose infusion. The Fast line had a significantly lower concentration of plasma glucose and higher plasma insulin than the Slow.

	Fast	Slow
Glucose	598.1	677.1*
Insulin	85.3	63.7*

Table 6.4 Area under the glucose curve and insulin response curve after a glucose tolerance test (P<0.05).

6.3.3 Carcass composition

Some of the carcass and noncarcass parameters measured at slaughter are shown in Table 6.5.

	Fast	Slow
Body gain (kg)	24.7	24.7
Carcass protein (%)	33.3	36.0
Carcass fat (%)	55.9	52.6*
GR (mm)	15.7	12.1*

Table 6.5 Various carcass and noncarcass parameters measured from sheep with High and Low responses to glucose (P<0.05).

Thus, selection for Slow glucose clearance was associated with significantly less carcass fat. Carcass protein was not significantly different. Thus while animal growth was the same between the two lines (with respect to weight gain) the body composition indicates that the Slow glucose clearance animals are leaner than the Fast.

6.3.4 Meat characteristics

Figures 6.4 and 6.5 show pH and kgf of chops (loins) from lambs at various times post-slaughter.

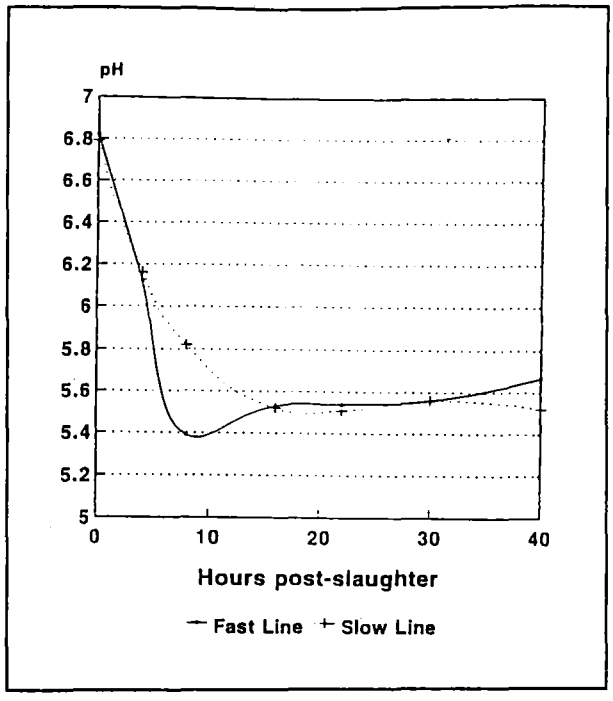

Figure 6.4 Average pH profile of loins from High and Low sheep lines up to 50 hours post-slaughter.

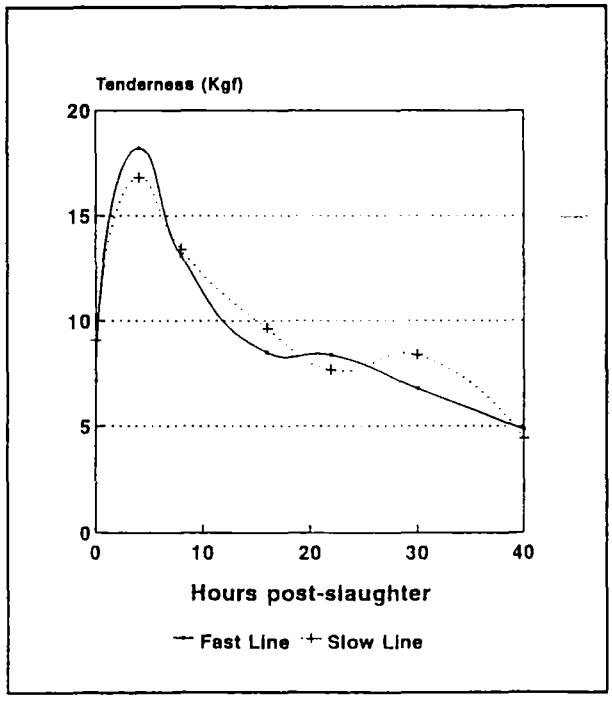

Figure 6.5 Average tenderness profile of loins from High/Low sheep lines post-slaughter (50hrs).

The tenderness and pH profiles shown in Figures 6.4 and 6.5 are typical of meat stored post-slaughter. There are no significant differences between High and Low lines of sheep with respect to either pH or tenderness.

6.4 Discussion and conclusions

The experiments described in this chapter were motivated by the observation that sheep with differences in glucose tolerance differed in body composition and certain metabolic parameters. Specifically, sheep with Slow glucose clearance (high T½) had a lower fat content and a greater percentage of protein (ie. were leaner) than their Fast counterparts. This agrees with earlier experiments using the same sheep lines (Francis 1990). Insulin is possibly the most important peptide hormone in the partitioning of nutrients between tissues (Prior *et al.*, 1982; Bickerstaffe 1993; Hart 1983; Brockman *et al.*, 1986). It seems reasonable to suggest that the dissimilarity in glucose tolerance and body composition may originate from differences in the insulin status of the two lines of animals. Francis concluded that following a glucose challenge, Slow animals release less insulin than the Fast. This is responsible for the slower glucose clearance and is possibly due to differences in the sensitivity of the pancreas to glucose. In addition, in the basal state, the Slow animals possessed more insulin receptors than Fast. This was demonstrated by the higher turnover rate, and the greater glucose utilization and the sensitivity to insulin of the Slow animals. The question remains, how do differences in insulin status affect changes in body composition? This chapter is concerned with exploring the possibility that insulin modifies protein degradation in skeletal muscle by altering the activity of the calpain proteases and their inhibitor, calpastatin.

6.4.1 The effects of insulin in ruminants

Insulin is primarily involved in the maintenance of glucose homeostasis in all animals and has anabolic effects on metabolism (Brockman 1986a). It is apparent from a number of studies that ruminant animals are less sensitive to the effects of insulin than nonruminants (McDowell 1983). The increment in plasma insulin resulting in a 50% reduction in glucose production is 50-60μU ml^{-1} in ruminants. The comparable value in humans is 30μU ml^{-1} (Brockman *et al.*, 1986). It is thought that these differences probably result from differences in intermediary metabolism particularly that of glucose (Janes *et al.*, 1985). In sheep, insulin promotes the absorption of glucose into peripheral tissues such as fat and muscle (McDowell 1983; Brockman *et al.*, 1986) resulting in a depression of plasma glucose (Call *et al.*, 1972). In 1989, Carter *et al.*, (1989) selected pigs on the basis of backfat depth and bred two distinct lines, one with high backfat depth and the other with low. Following a glucose challenge, the high backfat animals had significantly elevated plasma glucose

levels over a time period of 2.5 - 7.5 minutes and higher plasma insulin concentrations. Because the higher backfat animals were less able to remove plasma glucose, despite having greater plasma insulin levels, the authors suggested that this may be due to a reduction in the sensitivity of peripheral tissues to insulin. Indeed, Weekes (1986) reported that significant differences exist in the extent to which insulin can promote glucose absorption into different tissues and that skeletal muscle is the most sensitive tissue.

Liver gluconeogenesis is the main source of glucose in ruminants. Ruminant animals are forced to use sources of insoluble carbohydrate, such as starch, pectin, cellulose and hemicellulose, for their primary energy source (Atlas 1989). Formation of mutualistic relationships with rumen bacteria permits fermentation reactions converting these substrates into fructose-6-phosphate (Brown 1988). This in turn is converted to pyruvate and then to volatile fatty acids (VFA's) such as acetate (Matthews *et al.*, 1990; Brockman *et al.*, 1986). The VFA's are used as gluconeogenic substrates, and in the liver are metabolised into glucose (Willis 1990). Insulin inhibits liver gluconeogenesis and reduces glucose production (McDowell 1983; Brockman *et al.*, 1986). Some substrates for gluconeogenesis may be derived from mobilised skeletal muscle. The release of these glucogenic substrates is inhibited by insulin (Hart 1983). Glucagon opposes the effects of insulin and promotes gluconeogenesis (Brockman *et al.*, 1986; Hart 1983). It may be that regulation of gluconeogenesis relies upon the ratio of insulin to glucagon rather than on the level of either hormone alone (McDowell 1983).

Diet has significant effects upon glucose metabolism and most insulin effects can be altered by changing the nutritional status of animals. In general, fed animals are less responsive to changes in insulin than their fasted counterparts, although it has been reported that in the fed state, insulin levels in a sheep hindlimb rise and adjustments in metabolism occur (Prior *et al.*, 1984). However, this is questioned by Oddy *et al.*, (1987) who observed no variation in protein metabolism of fed lambs infused with insulin. Conditions of undernourishment, however, lead to a net loss of skeletal muscle in the hindlimb (Lomax 1987) resulting from a 62% decrease in protein synthesis and a 29% increase in degradation. Refeeding *ad lib* restores the original protein balance by depressing protein degradation and promoting synthesis. Oddy *et al.*,(1987) reported that fasting induces net protein loss but observed that the reaction of fasting lambs is to initially reduce protein degradation and later depress synthesis.

The three main components of the adult animal are bone, skeletal muscle and fat. Fat has the most variable growth rate (Weekes 1983). Insulin has the potential to modify the hypertrophy (growth) of adipocytes but not their hyperplasia (proliferation). In keeping with the anabolic effects of

insulin, it is apparent from some studies that elevated insulin levels result in lipogenesis (McDowell 1983) and antilipolysis (Brockman *et al.*, 1986). Genetically leaner breed of pig have been shown to be less sensitive to the antilipolytic effects of insulin (Wood *et al.*, 1977). The stimulatory effect of insulin on lipogenesis was lower in lean rats compared to obese (Carnie *et al.*, 1979). As already noted, the experiments performed by Carter *et al.*, (1989) that high backfat sheep had significantly elevated plasma insulin as well as fat content. However, these observations are doubted by Vernon (1986) who reported that although adipocytes in sheep possess insulin receptors with similar quantities and properties to those of the rat, ruminant tissues have failed to respond to insulin. He suggests that the duration of insulin exposure is important in eliciting physiological responses, and that effects from chronic exposure are more perceptible. Weekes (1986) concurs and indicates that it is difficult to demonstrate, *in vitro*, stimulatory or antilipolytic effects of insulin on tissue slices or isolated adipocytes. The available evidence caused Prior *et al.*, (1982) to suggest that glucose may be more important than insulin in the stimulation of lipogenesis.

Protein synthesis is thought to be positively influenced by insulin. This is determined by measuring the flux of amino acids within a closed metabolic system such as the hind leg. This is a region of the animal where blood flow can be monitored accurately. By quantifying amino acids, such as leucine, valine, isoleucine, lysine and tyrosine (Prior *et al.*, 1982) the protein status of an animal can be estimated. A net reduction in the plasma content of amino acids leaving the limb indicates that partitioning of amino acids into protein has occurred, and a increase suggests degradation. Insulin's anabolic effects on protein metabolism include stimulation of amino acid uptake as indicated by a fall in plasma levels (Call *et al.*, 1972; Brockman *et al.*, 1986), a rise in protein synthesis (Crompton *et al.*, 1987) and an increase in RNA/DNA synthesis (Buttery 1983) by insulin. In sheep this stimulation is particularly apparent in skeletal muscle. However, Oddy *et al.*, (1986) reported that several studies have expressed difficulty in demonstrating these positive effects of insulin in sheep. For example, in lambs 5-6 weeks old, there was no correlation between plasma insulin and protein synthesis. This maybe due to the insulin resistance during the early growth phase. However even in weaned lambs there is a very poor correlation (Vincent 1984 - Cited by Oddy *et al.*, 1986).

The levels of circulating urea provide evidence of the degree of protein degradation that has taken place (Hill *et al.*, 1989) since urea is a nitrogenous byproduct of protein degradation. Call (1972) reported that in sheep given an acute infusion of insulin, the plasma urea concentrations did not change. This observation is supported by Weekes (1982) who infused sheep with insulin and glucose for 9 days but observed no significant change in nitrogen excretion despite a modest rise in insulin levels. However, in the high and low backfat sheep, the high backfat animals (high insulin)

demonstrated significantly higher plasma urea levels (Carter *et al.*, 1989). It is possible, therefore, that for insulin to modify proteolysis and hence urea levels, a chronic disruption to insulin status is required. It is also possible that the lower plasma urea concentrations apparent in the lean animals (low backfat) may indicate more efficient use of amino acids for protein synthesis.

6.4.2 Assessment of the Calpain system

The first objective was to determine if techniques used in this experiment were successful in separating the components of the calpain system. Figure 6.1 provides the enzyme activity profile from one of the 20 animals examined. The assay detected proteolytic activity which corresponded to that expected for calpain I and II. This indicates that the activities of these enzymes have been quantified effectively from the tissue samples. There is some irregularity with the location of the activity (data not shown) but this is probably due to using different fraction collector machines. However, another irregularity that cannot be explained is the significant variation in calpain activities, between and within lines of animals. Tables 6.2 and 6.3 show that the standard variations are proportionately high (eg., the SD for calpain II both in Fast and Slow sheep lines is approximately 50% of the mean). It is difficult to explain this without more information, but there are a number of possible reasons. One possibility is that the initial 64 sheep were killed on three different days and environmental factors such as temperature, feed, and stress varied between the days and led to fluctuations in protease levels. However, analysis of variance to compare the calpains and calpastatin among the three kill days, showed no significant trend, indicating that the variable kill day did not have a significant effect on the calpain levels. A second possibility is that the differences vary according to the sampling position of the muscle. Work from this laboratory has suggested that there may be variations in enzyme levels within the *longissimus dorsi* (unpublished data) and this could well account for the observed differences. A final possibility is that the slaughter of the animals was carried out at the Templeton Research Station rather than under industrial conditions. Efforts were made to ensure accurate repetition of the processing at Templeton but time delays were inevitable. Following the observations made in Chapter 5, care was taken to remove samples at the same times relative to exsanguination. However some variation was observed and may contribute to the observed variation in enzyme activity. All protocols at Lincoln were uniform. Other work carried out in this laboratory has also found significant variations in enzyme levels from similar sheep even when industrial processing was used. This suggests that the observed variation is an inherent variation and that lowering of the standard deviation could be achieved only by sampling more animals. Significant variations in calpastatin levels was apparent.

It has been reported in this trial that sheep with a fast insulin response have a lower percentage of whole body protein. Although not measured in this experiment, Francis (1990) reported that urea concentrations were elevated in the Fast line of sheep. These two observations suggest that protein degradation may play a key role in nutrient partitioning into skeletal muscle. For this reason the calpain proteolytic system was measured in both selection lines. Significant differences were found between the selection lines with respect to both calpain enzymes and their inhibitor. Calpain I and II were significantly ($P<0.05$) higher and calpastatin significantly ($P<0.05$) lower, in the Fast than the Slow line of sheep. Thus it would appear that selection for glucose tolerance selects for calpain mediated proteolysis. This has important implications for developing a better appreciation of ruminant metabolism. Because control animals were not included in this trial, it is difficult to know whether the reported enzyme levels are elevated or depressed relative to a normal animal. For the purposes of this discussion I will assume that control enzyme levels would lie between the values reported here.

Other studies have revealed a positive correlation of plasma insulin to urea (Francis 1990; Carter *et al.*, 1989; Bremmers *et al.*, 1988), where insulin is chronically elevated. In this experiment it seems reasonable to suggest that this could be due to increased calpain activity.

6.4.3 Glucose tolerance testing

The trends observed in Figures 6.2 and 6.3 are entirely consistent with those reported by Francis (1990). A higher T½, in the Slow animal line, indicates a slow glucose clearance. This could be due to differences in the sensitivity of the pancreatic ß cells to glucose, and/or the peripheral sensitivity of tissues to glucose. Accurate assessment of basal plasma insulin and glucose was not included in this trial, but in earlier work Francis reported no significant differences in insulin levels. But elevated plasma glucose in the Slow animals. Raised plasma insulin levels appeared to be notably lower in the Slow animals compared to Fast at 10, 20 and 40 minutes after a glucose infusion and divergence was still apparent at 90 minutes. This deviates from Francis (1990) who reported that after 60 minutes the difference had almost disappeared. However, she does not specify the concentration of glucose employed in her experiments and it is possible she infused less glucose/kg thus making the reestablishment of basal glucose and insulin swifter. Overall, the plasma insulin levels are lower in the Slow than Fast animals. Concordantly, glucose levels are lower in the Slow than Fast sheep following glucose infusion with the most obvious differences being apparent at 10, 20 and 40 minutes.

6.4.4 Body composition

Selection of sheep on glucose tolerance has resulted in phenotypic differences other than inherited T½ parameters. From Table 6.5 it is clear that sheep selected for Slow glucose clearance have a significantly reduced fat content and GR (subcutaneous fat) depth. Whole body protein content was elevated in the sheep but not significantly. There was no whole body weight gain differences between lines, so any partitioning imbalance is compensated by another. Thus, it would appear that the Slow sheep are leaner than the Fast. This conclusion concurs with that found by Francis (1990). Differences in body composition usually result from modification of peripheral tissue metabolism. It has already been noted that the Slow line of sheep have depressed levels of calpain proteases in skeletal muscle. This could account for the greater percentage protein and illustrates that the changes in the protein degradation system rather than protein synthesis could be contributing to the differences in the observed net protein deposition. The higher fat content of the Fast line of sheep is possibly due to augmented insulin response.

6.4.5 Meat quality

There are no significant differences in pH or tenderness between the two lines of sheep. The trends in Figures 6.4 and 6.5 are consistent with those seen in meat from nonselected animals. It may be anticipated that the Fast sheep, with their higher protease and lower inhibitor levels, may have tenderised sooner then the Slow. There were no apparent tenderness differences. Only during the 8-30 hour post-slaughter storage meat was there a tendency for the Fast sheep to be slightly tougher than the Slow. The final tenderness (Kgf) at 627 hours post-slaughter showed no differences between the lines.

6.5 Summary

Leanness is an important quality characteristic in meat animals, and techniques to improve leanness have potential commercial applications. In particular, physiological markers for genetic merit such as T½ for lean meat could reduce errors of prediction and permit the selection of breeding stock. Several studies have endeavoured to support this approach (Carter *et al.*, 1989; Bremmers *et al.*, 1988). The observations made in this experiment acquiesce with the observations made by Francis. Specifically the Slow line of animals (Lean) had a higher area under the glucose clearance curve, a smaller area under the insulin response curve, reduced carcass fat, and increased protein. Possibly coupled with this latter observation was a significant reduction in the muscle protease activities (Calpain I and II) and an increase in calpastatin activity. It is possible to hypothesise that there is

an association between the changes in calpain activities and net protein deposition. There were no differences in pH or tenderness in *longissimus dorsi* muscles between the two lines of animals.

The results raise a number of questions. Firstly, is the calpain status of the fat animals responsible for their lower protein content? Why would their metabolism seek to partition nutrients into fat rather than protein when feed is not a limiting factor? Why do animals with measurably greater levels of insulin (post glucose challenge) have less whole body protein when insulin supposedly promotes protein accretion? How does the selection for glucose tolerance lead to the altered calpain status? Is it because insulin stimulates calpain gene expression (just as it does other genes (Meisler *et al.*, 1989))? Or has the selection for genes regulating glucose tolerance selected genes regulating calpain expression? Weekes (1986) states that the effects of insulin on phenotypic traits (eg fat/protein accretion) must be treated with care, and that insulin must not be considered in isolation but rather in relation to levels of other circulating hormones (eg growth hormone, glucagon), metabolites and neural inputs. Also, why do differences in calpain status not reflect in differences in other meat quality characteristics eg., tenderness? In conclusion, this work has served to emphasise the complex nature of nutrient partitioning in ruminants. It has also demonstrated that selection of animals on one phenotypic quality can modify others. These associations are incompletely understood.

Chapter 7 :
Effect of growth hormone vaccine on calpain proteases and meat quality characteristics.

7.1 Introduction.

Improvement of animal production by administration of hormones is under pressure from the public and authorities as the image of 'hormone free meat' becomes increasingly appealing. Over the past 20 years immunological methods for manipulating animal growth have been researched, and various technologies are now available for experimental use. This trial investigates the technique of injecting sheep with an immunogenic fragment of ovine growth hormone, specifically the active site. In theory this region of the molecule should elicit an immune response in the treated animals, and polyclonal antibodies specific for this fragment of growth hormone will be raised. These antibodies subsequently bind to the active site of endogenous growth hormone and potentiate its biological operation. This response cumulates in augmented activity of the endogenous growth hormone and is reflected, physiologically, in increased muscle growth and adipolysis. Previously this technology has been shown to improve growth rate (Aston *et al.*, 1991) wool production (Spencer *et al.*, 1983) and milk production (Pell *et al.*, 1989) in sheep. Other parameters considered in this work are growth, body composition, muscle protease activities, meat pH and tenderness.

7.1.1 Growth hormone and growth

Body growth in mammals is regulated by a blend of genetic, nutritional and endocrine factors. The endocrine system has a major impact on both growth and development of an organism (Millard 1989). Growth hormone (GH) the is principal hormone associated with growth.

The growth promotive effects of GH were first recognised in the classic work of Evans and Simpson (1931) who demonstrated that chronic administration of crude bovine GH increased the weight gain of rats. Later it was identified that the weight gain arose from an increase in protein accretion and a decrease in fat deposition (Lee *et al.*, 1934). Since this time the effects of GH on repartitioning nutrients have been observed in a number of reports (Brockman *et al.*, 1986; Buttery 1983; Bass *et al.*, 1989).

The consequences of exogenous GH administration on protein metabolism appear to be primarily associated with postabsorptive use of nutrients (Boyd *et al.*, 1989). Frequently, the effects of GH are divided into two classes, termed 'direct' or fuel regulation and 'indirect' or growth stimulation effects (Ballard *et al.*, 1993) see Figure 7.1.

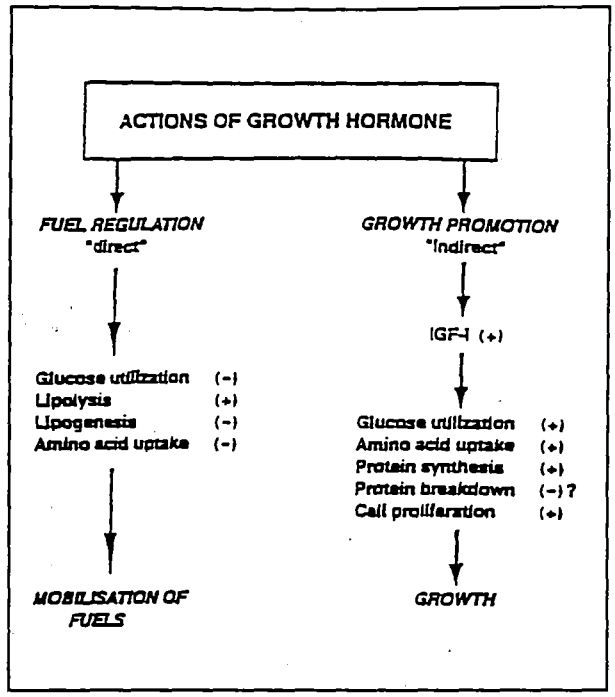

Figure 7.1 Schematic representation of GH effects on fuel regulation and growth promotion (From Ballard *et al.*, 1993).

Responses to fuel mobilisation include repression of glucose utilisation, lipogenesis, amino acid uptake and increased lipolysis. The important point is that these effects cannot be likened to growth; but represent wasting. The second series of responses (indirect) are induced by GH and lead to growth promotion. They include glucose and amino acid uptake, protein synthesis, cell proliferation, and possibly inhibition of protein degradation. Figure 7.1 shows that many of the GH effects are contesting. Thus the relative strength of either arm will determine the observed effect of GH. Generally, however, the overall effect of GH is to partition energy into growing tissues (Bass *et al.*, 1989). The following sections describe in more detail some effects.

Glucose uptake - GH is influential in maintaining glucose homeostasis in both ruminants and nonogastrics (McDowell 1983). However, whether its effects are direct (eg., promoting luconeogenesis) or indirect (eg., sparing peripheral glucose utilization) is unclear. What is pparent is that the relationship between GH and plasma glucose is negative. Bassett (1974) eported a rapid decline in GH following an equally rapid rise in insulin in lambs ingesting milk. urthermore, GH appears to antagonise some effects of insulin. For example increases in plasma

fatty acids are often witnessed following GH infusions. Further, when animals are selected on the basis of fatness (eg., backfat depth), a negative correlation with GH is typically seen (Weekes 1983).

Cell proliferation/ differentiation - GH promotes the conversion of fibroblast cells (undifferentiated cells) into mature adipocytes (Hausman *et al.*, 1989). This direct effect of GH is coupled with stimulation of IGF-I which then acts as a mitogen and promotes multiplication of the differentiated cells. This twin effect of GH is called the Dual Effector Theory (Bass *et al.*, 1989), and has also been witnessed in GH mediated chondrocyte and myoblast differentiation *in vitro* (Bass *et al.*, 1989; Spencer 1989). With respect to the latter cell type, however, Dayton *et al.*, (1989) argues that while chronic administration of GH promotes muscle accretion, there is no evidence to suggest that it promotes myogenesis or postnatal muscle growth. Dayton *et al.*, (1989) states that several studies have shown no effect of GH on proliferation or differentiation in cultured satellite cells, myotubes or embryonic myoblasts. IGF-I, however, has been shown to stimulate proliferation and differentiation in these cells. Therefore, rather than GH having direct effects on proliferation and differentiation it may be that much if not all of muscle growth is mediated through IGF-I in response to GH.

Protein synthesis and degradation - It is generally accepted that the main action of GH on protein metabolism is anabolic (Buttery 1983). For example, Snell dwarf mice are genetically hypopituitary, and the administration of bovine GH to these mice leads to a significant increase in muscle weight (Pell *et al.*, 1992). Seemingly this is due to a 50% increase in protein synthesis (Ks) as detected by the incorporation of radiolabeled phenylalanine into muscle. In turn, the stimulation was probably due to increased RNA content and efficiency of protein synthesis when expressed as grams of protein synthesised per gram of RNA per day. Martinez *et al.*, (1991) has also witnessed a significant increase in the rate of protein synthesis in response to GH. In this case GH infusion into fasted female rats produced a 25% increase in muscle Ks within 40 minutes. In times of energy deficit (such as during lactation) the role of GH is thought to preserve body protein by promoting protein synthesis and inhibiting proteolysis (Hart 1983). With respect to proteolysis, there is evidence to suggest that GH has the potential to effect nutrient partitioning by altering protease activity. Plasmin (an enzyme protease) is detectable in mammary tissues in late lactation and probably has a role in tissue remodelling (Bickerstaffe 1993). Both IGF-I and bovine GH inhibit plasmin secretion and in so doing alter the partitioning of nutrients between mammary and peripheral tissues. More direct evidence comes from a study where cathepsin A activity (units/gm) was significantly elevated (Martinez *et al.*, 1991) in response to GH. Further, in a study performed by Pell *et al.*, (1992) on GH treated Snell dwarf mice, the body protein content at death was less than expected from the increase in Ks brought about by GH. This implies that muscle protein degradation might have increased, and account for this difference.

Thus, the reported effects of GH on ruminants and non-ruminants are diverse. Most researchers agree that GH promotes lean growth in adequately fed ruminants. Certainly from the perspective of the meat industry, the most interesting and important potential of GH is the partitioning of nutrients away from fat into muscle tissue (Bass *et al.*, 1989). This experiment intends to measure changes in metabolism arising from the use of GH peptide, and its effects on the calpain system and other meat parameters (pH and tenderness).

7.2 Methods.

7.2.1 Animals

Sixty-four animals were maintained over the trial period at the Templeton Research Station, Canterbury. Animals were kept on pasture and weaned according to normal practice. There were no restrictions on feed consumption at any time. Slaughter of the animals took place in the last week of March 1993 when they were 32 weeks of age. For convenience, the animals were randomly allocated to one of three kill groups. The first group was slaughtered on Monday, the second Wednesday and the last on Friday.

7.2.2 Trial Design

The 64 animals were divided into two groups of 32 based on sex and into two further subgroups of 16 (treated and untreated). Allocation of animals into the treatment groups was random. The peptide used in this trial corresponded to a biologically active region of GH between amino acids 122-164. Peptide was administered in a single shot irrespective of live weight at 8, 12 and 16 weeks. These times were chosen to maximise GH activity at the time of fat deposition (16 weeks +). Control lambs were injected with Freund's adjuvant only.

7.2.3 Calpain system separation and assay

Calpains were prepared from 40gm sample of the *longissimus dorsi* muscle taken from 20 ram animals (10 Immunised, 10 Control), 20 minutes after death. A 5gm subsample of the muscle was trimmed of visible fat and connective tissue before being minced with scissors and a scalpel. The sample was then homogenised in 5 vols (25ml), of cold extraction buffer (100mM TRIS-HCl, 10mM ß-MCE, 10mM EDTA, pH 8.3). Homogenisation took place in a 250ml Waring Blender cup using a Waring Blender set on high speed. Three 30 second homogenisations were separated by cooling periods of 30 seconds. The homogenate was centrifuged at 15,000 rpm for 1 hour at

4°C, the supernatant decanted off and filtered through a washed glass wool/cheese cloth sandwich. The pH of the filtered extract was adjusted to 7.5 with 1M acetic acid and dialysed for 18 hours against dialysis buffer (40mM TRIS-HCl, 3mM EDTA, 10mM ß-MCE, pH 7.5) at 4°C. The extract was filtered through a washed, moist glass wool/cheese cloth sandwich and then loaded onto a DEAE-sephacel column (1.5 x 20cm) which had been equilibrated with elution buffer (40mM TRIS-HCL, 0.5mM EDTA, 10mM ß-MCE, pH 7.5). The column was washed with 250ml elution buffer and then the bound proteins eluted by a continuous gradient of NaCl in elution buffer from 25mM to 350mM (120ml of each).

A sample to be tested for calpastatin content was extracted from the sample following the centrifugation step described above. A 1ml subsample of the extract was boiled at 95°C for 15min before being spun at 11000 rpm for 5min. The resulting supernatant was assayed for calpastatin.

The activities of calpain I and II were determined using casein as a substrate. Briefly, 0.5ml of fractions 1-55 and 0.25ml of fractions 56-80 were combined with appropriate volumes of Ca^{2+} and EGTA casein (10mM TRIS-HCl, 1mM NaN_3, 10mM ß-Mercaptoethanol, 5mg/ml Casein, 5mM $CaCl_2$ or 10mM EGTA, pH 7.5) to bring the total volume up to 1.0ml. The mixture was incubated at 25°C for 1 hour and the reaction stopped by the addition of 1.0ml ice cold 5% TCA. Assay tubes were spun at 4000rpm for 15 min and the soluble digestion products in the supernatant measured spectrophotomerically at 278nm. One unit of calpain activity is defined as the amount of enzyme catalysing an increase of 1 absorbance unit at 278nm in 60min at 25°C.

Calpastatin was assayed by incubating a 250µl subsample of the soluble calpastatin fraction with a volume of calpain II possessing an activity of 0.25-0.30 units. Loss of activity in these tubes, when compared with control (calpain only) represents calpastatin activity.

7.2.4 Carcass measurements

Two weeks after the last immunisation, blood samples were collected from all animals for antibody assay. Animal live weight was measured from the beginning of the trial and every 3 weeks thereafter.

At 22 weeks the animals were intravenously injected with synthetic hGRF (human growth hormone releasing factor) at 0.3µg/kg live weight. Blood samples were collected at -15, 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes relative to the injection and assayed for GH.

At 26 weeks measurement of hot carcass weight and subcutaneous fat over the 12th rib at the GR position were made at Templeton. One half of the carcass was retained and frozen for subsequent

mincing and carcass analysis of total fat and protein.

After slaughter, loins from both sides of the carcass were cut into 40mm chops to yield 8 chops per animal. The chops were stored at 1°C and at 0, 4, 8, 10, 12, 22, 30, 40 and 627 hours post-slaughter, pH and tenderness were measured. Tenderness was assessed following standard procedures using a MIRINZ tenderometer (Quality Assurance for Tenderness MIRINZ technical report 872, ISSN 0465-4390). pH was measured with a Hanna HI9025C portable pH meter with an Orion Ross combination spear tip pH electrode (81-63).

7.2.5 Statistical analysis

Data was tabulated in Quattro Pro spreadsheets and analyzed by Minitab Statistical Software package. Data was analyzed using ANOVA and Students *t* Test.

7.3 Results

7.3.1 Calpain proteases and calpastatin

The separation and quantification of calpain I, calpain II and calpastatin was successful. Table 7.1 summarises the activities of the separated enzymes.

Animal	Immunised			Control		
	Calpain I	Calpain II	Calpastatin	Calpain I	Calpain II	Calpastatin
1	0.52	1.24	0.45	1.12	0.72	0.37
2	0.86	1.00	0.14	1.07	0.52	0.37
3	0.69	0.88	0.07	0.99	0.30	0.27
4	0.57	0.60	0.10	0.72	0.40	0.21
5	0.43	0.88	0.18	0.32	0.24	0.15
6	0.55	2.42	0.30	1.26	1.12	0.07
7	1.12	1.38	0.04	1.03	0.78	0.10
8	1.42	0.86	0.20	0.51	0.50	0.09
9	1.52	0.58	0.03	0.83	1.06	0.03
10	0.91	1.18	0.12	1.46	0.94	0.09
Average	0.86 (±0.4)	1.10 (±0.5)	0.16 (±0.13)	0.93 (±0.3)	0.65 (±0.3)*	0.17 (±0.12)

Table 7.1 Summary of calpain I, calpain II and calpastatin activities (Units/gm) from LD muscle of immunised and control rams. Enzymes were extracted using DEAE sephacel and a linear NaCl gradient, assaying followed the standard procedure described earlier ($p<0.05$).

Calpain II appears to decrease in the GH immunised animals compared to control. Calpain I and calpastatin were unaffected by the treatment.

7.3.2 Antibody assay

GH specific antibodies were assayed for 2 weeks after the last peptide injection. Figure 7.2 shows the response of GH antibody to the peptide immunisation.

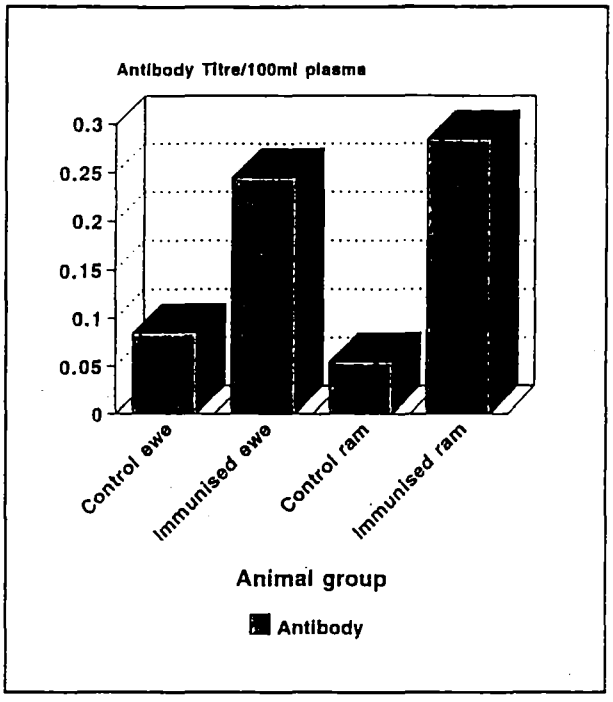

Figure 7.2 Growth hormone antibodies produced in response to immunisation with GH peptide in different treatment groups.

As anticipated, immunisation stimulated the production of significantly ($p<0.001$) higher levels of antibodies in the treated group. Specifically, the values were 0.081 (±0.006) and 0.26 (±0.015) for the control and immunised groups respectively. There did not appear to be any effect of sex on the antibody titre production.

7.3.3 Growth rates

The live weights of all the animals were measured at birth, and approximately at 4 week intervals thereafter. The growth trends are shown in Figure 7.3

There was no significant effect of immunisation on growth rates during the experimental period. However, it did appear towards the end of the trial that the immunised animals were growing at a slightly greater rate than their control counterparts. Males grew faster than the females ($p<0.05$, data not shown). This graph has been calculated to remove the effect of sex.

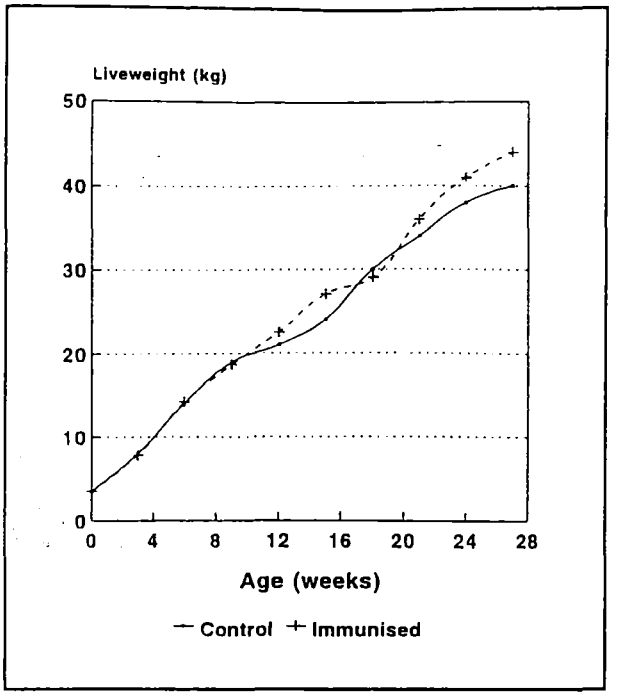

Figure 7.3 The effect of immunisation on the growth rates (live weights) of lambs over the experimental period.

7.3.4 hGRF challenge

Human GRF was infused into all animals at 22 weeks of age. Figure 7.4 shows the effect of this on plasma GH between the two animal treatments.

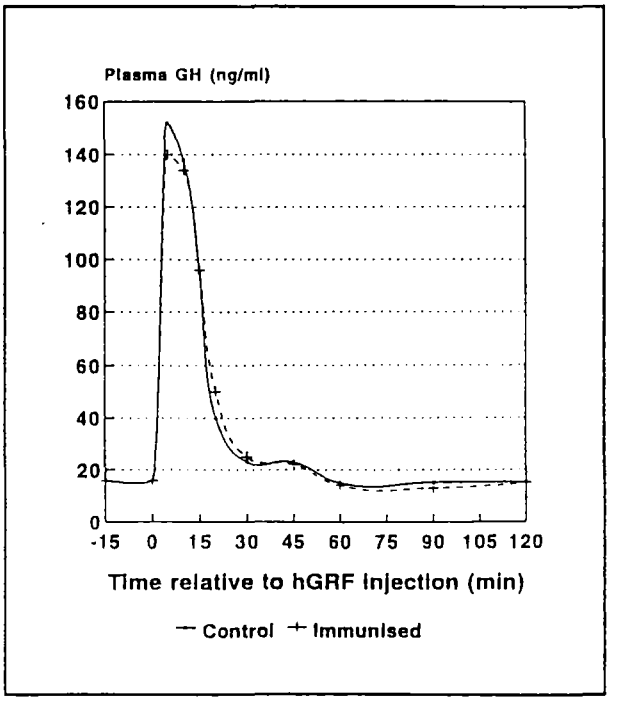

Figure 7.4 Plasma growth hormone response to hGRF, and the effect of immunisation with GH peptide.

As was expected, infusion with hGRF induced an increase in circulating GH. Immunisation with GH peptide did not appear to effect this response as there was no significant difference between the two response curves.

7.3.5 Carcass composition

Body composition parameters were measured after death, these are summarised in Table 7.2.

	Control	Immunised
Body Gain (kg)	24.4	25.0
Carcass Protein (%)	34.9	34.4
Carcass Fat (%)	53.8	54.6
Kidney Fat (gm)	197	239*
GR (mm)	13.3	14.5
Omental Fat (gm)	516	572

Table 7.2 Comparison of various carcass and noncarcass parameters ($p<0.05$).

Immunisation against GH had no effect on total carcass composition. There were, however, significant elevations in kidney fat. GR and omental fat were also higher in the immunised animals but not to a significant degree.

7.3.6 Meat characteristics

The pH and tenderness (Kgf) of the LD muscle were measured at various times post-slaughter. Figures 7.5 and 7.6 illustrate the post-slaughter changes in pH and tenderness respectively.

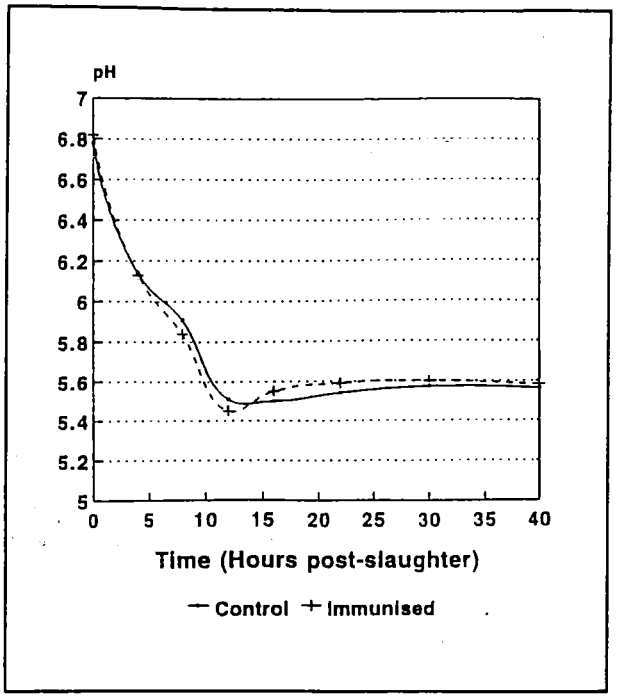

Figure 7.5 The effect of immunisation on the pH profile of LD muscle stored up to 40 hours post-slaughter at 4°C.

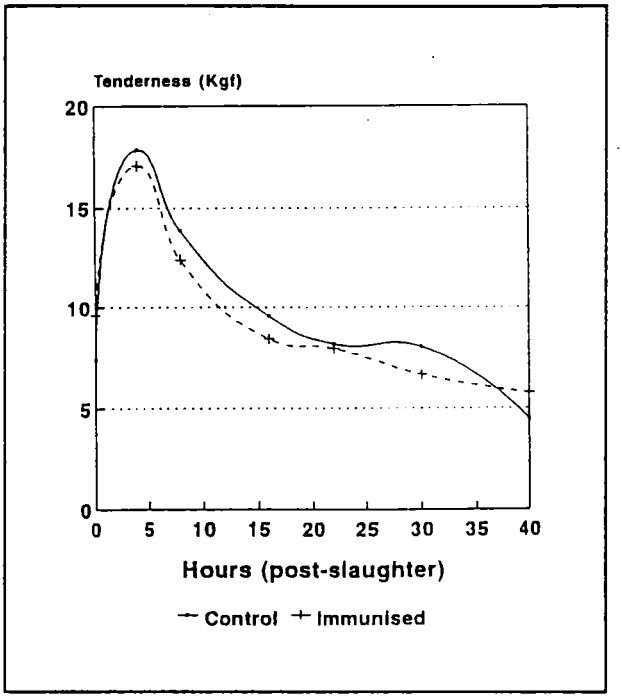

Figure 7.6 The effect of immunisation on tenderness of LD muscle stored up to 40 hours post-slaughter at 4°C.

Both Figures 7.5 and 7.6 comply with the expected trends in pH and tenderness post-slaughter. There was no effect of immunisation on the rate of change or final pH or tenderness values.

7.4 Conclusion and discussion

The use of monoclonal antibodies to modify animal growth and production has been successful in some experiments (Holder *et al.*, 1988; Aston *et al.*, 1991/1986; Pell *et al.*, 1989). This experiment investigated whether the polyclonal antibodies produced in response to GH active site would be similarly successful. There are important advantages of this system of growth manipulation over other biotechnologies such as direct infusion of hormones (GH, IGF-I or GRF) or genetic manipulation (selective breeding or transgenics). Firstly, once biologically active topographic regions of GH are found and produced as commercial peptides, relatively few immunisations (≈3) will be required to produce a long lasting response. This is suitable from a price and labour perspective, and individual immunisations are practical. But possibly more importantly, the public's perception of this approach is that it offer natural potentiation of an endogenous hormone product. In contrast, introduction of artificial products, whether it be hormones or DNA, is often seen by consumers as potentially harmful, and consequently discouraged by authorities.

A modification of growth rate is possibly the most telling sign of the success of this work. However, this trial failed to establish any effect of the peptide immunisation on live weight gain during the trial period. This differs from other published works where growth rate has been successfully modified. For example, in a study by Holder *et al.*, (1985) Snell dwarf mice treated with a hGH/mAb (EB1) complex over a 6 week period displayed a significantly ($p<0.001$) greater weight gain than mice receiving hGH alone. A similar report was made by Wallis *et al.*, (1987) who infused a bGH/mAb complex into hypophysectomised rats. Both reports showed that the weight gain is dependent upon the mAb used. Weight gain is not an exclusive response to GH modifications. For example, immunisation against SRIF in lambs (Spencer *et al.*, 1983) and IGF-I in guinea pigs (Kerr *et al.*, 1990) have also produced weight gain. Importantly, the studies referring to GH modified growth rates were both performed in GH deficient models, and consequently the potential for growth modification by GH/mAb is high. This differs from the current experiments on sheep with normal GH levels. It may be that polyclonal antibody induced GH potentiation in sheep with adequate feed and no GH deficiency show a less conspicuous response to GH than in trials where GH is depressed.

To ascertain whether GH activity had been successfully potentiated, a hGRF challenge was carried out. Examination of the GH response to hGRF is often when the GH axis has been modified, Dubreuil *et al.*, (1989/1990). If polyclonal antibodies with potentiating ability had been raised against the peptide, then either a heightened or prolonged GH response to hGRF may be expected. Unfortunately, neither was seen and the plasma GH response to hGRF was not significantly

different between the immunised and control animals. Incompatibility between hGRF and the ovine GH axis is a possible explanation. However other studies have reported hGRF's capacity to augment GH in nonhuman systems. For example, in pigs (Pommier *et al.*, 1990) and cattle (Moore *et al.*, 1992). Accordingly it seems unlikely that use of hGRF in ovine GH stimulation should be capricious. However, this is not a conclusive test since the mechanism of polyclonal antibody enhancement may be via a prolonged receptor association (Holder *et al.*, 1987), the "restriction hypothesis" (Aston *et al.*, 1991), or the induction of conformational changes in the GH molecule resulting in elevated activity (Holder *et al.*, 1991).

Several other suggestions have been made to explain the observed results. It is conceivable that the trial was terminated too soon. At 27 weeks the growth rates were beginning to diverge (Figure 7.3) and prolonging the trial may have enable a statistically significant deviation to be seen. It is also possible that the topographical region where the peptide was modeled (aa 122-164) is not biologically active. In a study performed by Lewis *et al.*, (1975) the controlled digestion of hGH eliminated residues 138-147 and resulted in increased growth activity and weight gain. This suggests that a significant portion of the peptide used in this work actually has a depressive effect on GH activity. However, more recent work by Aston *et al.*, (1991) refutes this and asserts that extensive peptide synthesis has revealed two loop regions (sequences 35-53 and 134-154) which possess GH-like activity. The second of these regions (134-154) is contained within our peptide, and therefore advocates its potential for activity. Furthermore, the antibodies raised against the peptide may have been neutralised by a secondary factor. This proposal could be appraised by examining whether plasma IGF-I concentrations were greater in the immunised animals. This would also substantiate whether the region of GH used was biologically active in the stimulation of IGF which is thought to mediate the effects of GH. Studies in hypophysectomised rats (Wallis *et al.*, 1987), have shown an increase in IGF-I levels following bGH/mAb administration. However, the fact that kidney fat and calpain II levels were significantly changed by immunisation suggests that neutralisation of mAb activity did not occur.

With respect to muscle quality, it is not clear whether the GH axis has affected parameters such as pH and tenderness, although positive effects on protein deposition and depression of protein degradation have been previously described (Doherty 1992; Oddy *et al.*, 1987). Indeed, where a hGRF analogue was infused into pigs, pH was not affected although other parameters, such as protein and muscle weight, showed differences (Pommier *et al.*, 1990). This observation has been affirmed in these experiments where no change in pH or tenderness were observed, despite a significant reduction in calpain II occurring after immunisation. This decline was not reflected in any significant improvement in tenderness, which is not surprising since calpain I is considered to be more crucial in establishing tenderness.

7.5 Summary

It would appear from this work that the immunisation of normal sheep with a GH peptide (122-164) does induce an immune response. However, the polyclonal antibodies generated in response to the peptide region do not have the predicted capacity to potentiate the actions of endogenous GH. The reasons for this failure are unclear although there is little evidence to indicate that the fault lies with the peptide or the immune response. Possibly the largest difference between this trial and others, is that the animals used in the trial were typical with respect to endogenous GH levels. As a result of the unrestricted growth in the early stage of life, any slight potentiation of growth may only be reflected over the long term. Modification of the calpain system by the immunisation technique is interesting but there was no effect on any meat quality parameter suggesting its usefulness is limited. Nevertheless, it does indicate that immunomodulation has the potential to manipulate the calpain system, and this may be investigated further.

Chapter 8

General discussion and conclusions

; thesis has described a variety of approaches used for the extraction of calpain proteolytic system ponents. In the results presented here only those employing DEAE-sephacel have been reliable. As a lt, a DEAE-sephacel protocol based upon methods reported elsewhere has been developed and loyed. Some reservation exists, however, when comparing absolute results ((eg., units of enzyme per ı) determined by this method, with results seen in other research papers. Specifically, this is because ls of enzyme appear to be lower in these experiments than reported elsewhere. A number of anations exist for this. It is likely that there is considerable animal variation in enzyme activities and ;asing sample size may be advantageous. Differences in post-mortem sampling times can have ıatic effects on enzyme yields. This conclusion is based upon chapter 5 results which established a l for calpains to decline post-mortem. Finally dissimilarities in laboratory protocols may contribute to rences in reported enzyme levels. Little attempt was therefore made to compare enzyme values ;tly to other work. However, standardisation upon a single protocol means that results contained in thesis are comparable. Future experiments would benefit from modifying this protocol so that ɔarisons may be made universally.

ı attempt to better understand regulation of enzyme levels in the calpain system, this thesis included a which attempted to modify the GH axis in lambs. Infusion of the active site of GH produced a ;lonal immune response, but failed to generate a change in growth of the treated lambs. Similarly, ffects on calpain or tenderness were observed. Some data from this trial (growth rate curves) suggest ı delayed effect may have occurred and that prolonging the trial could have demonstrated a treatment t. However, this remains unproven. It might be interesting to experiment with antibody mediated in potentiation with respect to the calpains. A technique whereby calpain activity *in vivo* could be ıented or repressed would provide a useful experimental model, and may have commercial ;ation.

thesis has presented evidence to suggest that lambs differing in their response to glucose (glucose ınce rate), also differ in the levels of calpain I, II and calpastatin. Hormonal status may, therefore, some regulatory capacity over the calpain system. It is possible also that the lower protein content ved in slow clearance animals is due to elevated calpain I and II, and reduced calpastatin levels. A

: animal role for the calpains has not yet been discovered, and it would be interesting to establish a hnique which could quantify the calpains *in vivo*. This could then answer the question raised by this erence ie., whether the calpain system is involved in nutrient partitioning, and whether it is regulated the endocrine system. The differences in calpain levels between the lines of sheep did not correlate h a difference in meat tenderness. Other researchers have performed experiments which give umstantial evidence of a role for the calpains. While evidence certainly supports this association (eg., bition of calpain leads to tough meat, myofibrillar breakdown occurs in the presence of calpain) it / be difficult to prove *in vivo* as the process of tenderisation is multifactorial. Nevertheless, research ld be directed towards screening different animal breeds and attempting to correlate enzyme levels ı meat quality. Additionally, selective breeding may be able to generate high and low enzyme level :p for experimentation, and development of an understanding of the genetic basis for enzyme variation d prove useful for biotechnologies employing DNA manipulation.

Appendix 1

Method 1 (Koohmaraie 1990b)

5gm muscle sample
↓
Trim off fat and connective tissue
↓
Homogenise in 3vols Extraction Buffer:
(50mM TRIS, 10mM EDTA, 10mM ßMCE, pH 7.8)
↓
Centrifuge (15000rpm, 15min)
↓
Filter through glass wool/cheese cloth
↓
Adjust pH to 7.5 with 1M Acetic acid
↓
Reduce conductivity to equal Equilibration buffer:
(50mM TRIS, 1mM EDTA, 10mM ßMCE, pH 7.5)
use ice cold distilled/deionised H_2O
↓
Centrifuge (32000rpm, 60min)
↓
Load onto DEAE-Sephacel column
(1.6cm x 40cm)
↓
Elute with linear gradient 0-400mM NaCl in Equilibration buffer
(95ml each)
↓
Collect 64 x 3ml fractions

Appendix 2

Method 2 (Wheeler & Koohmaraie 1991a)

5gm muscle sample
↓
Trim of fat and connective tissue
↓
Homogenise in 10vol Extraction buffer:
(50mM Sodium Acetate, 10mM ß-MCE, 10mM EDTA,
0.2% Triton X-100, pH 5.8)
↓
Centrifuge (32000rpm, 60min)
↓
Filter through glass wool/cheese cloth
↓
Adjust pH to 7.5 with 1M NaOH
↓
Dialyse for 20 hours against Dialysis buffer:
(20mM TRIS, 5mM EDTA, 10mM ß-MCE, pH 7.5)
↓
Centrifuge (32000rpm, 60min)
↓
Filter through glass wool/cheese cloth
↓
Load onto DEAE-Sephacel
(1.5cm x 20cm)
previously washed with Equilibration buffer:
(40mM TRIS, 0.5mM EDTA, 10mM ß-MCE, pH 7.45)
↓
Elute Calpastatin and Calpain I with linear gradient
from 25-200mM NaCl in Equilibration buffer (90ml each)
↓
Elute Calpain II with 400mM NaCl in
Equilibration buffer (60ml)
↓
Collect 72 x 3.3ml fractions

Appendix 3

Method 3 (Thomson *et al.*, 1992)

5gm muscle sample
↓
Trim off fat and connective tissue
↓
Homogenise in 6vol Extraction buffer:
(40mM TRIS, 10mM EDTA, 0.2% Triton X-100, 10mM ß-MCE, pH 7.5)
↓
Centrifuge
↓
Filter through glass wool/cheese cloth
↓
Load onto 6ml DEAE-Sephacel
(1.0cm x 10cm)
previously washed with Buffer A:
(40mM TRIS, 0.5mM EDTA, 10mM ß-MCE, pH 7.5)
↓
Elute Calpastatin with 6vols of 6ml Buffer A + 100mM NaCl
↓
Elute Calpain I with 6vols of 6ml Buffer A + 200mM NaCl
↓
Elute Calpain II with 6vols of 6ml Buffer A + 400mM NaCl

Appendix 4

Method 4 (Golpalakrishna *et al.*, 1985)

1gm muscle sample
↓
Trim off fat and connective tissue
↓
Homogenise in 5vol Extraction buffer:
(50mM TRIS, 1mM EDTA, 10mM ß-MCE, 150nM Pepstatin A, pH 7.5)
↓
Centrifuge (15000rpm, 60min)
↓
Add 10µl 1mM Leupeptin
↓
Add 1ml Phenyl Sepharose washed with 0.25M NaCl in Buffer A:
(20mM TRIS, 0.1mM $CaCl_2$, 10mM ß-MCE, 20µM Leupeptin, pH 7.5)
↓
Shake mixture end over end for 5min
↓
Add 0.3ml 5M NaCl
↓
Shake mixture end over end for 5min
↓
Add 0.2ml 0.1M $CaCl_2$
↓
Shake mixture end over end for 10min
↓
Pour into Column
(0.8cm x 4.0cm)
↓
Wash with 2ml Buffer A + 0.25M NaCl
↓
Wash with 2ml Buffer A
↓
Wash with 2ml Buffer A - Leupeptin
↓
Elute Calpain II with 4ml 0.1M NaCl in Buffer B:
(20mM TRIS, 1mM EGTA, 10mM ß-MCE, pH 7.5)
↓
Elute Calpain I with 2ml Buffer B

Appendix 5

Method 5

1gm muscle sample
↓
Trim off fat and connective tissue
↓
Homogenise in 5vol Extraction buffer:
(50mM TRIS, 1mM EDTA, 10mM ß-MCE, pH 7.5)
↓
Centrifuge (15000rpm, 60min)
↓
Add 1ml Phenyl Sepharose washed with 0.25M NaCl in Buffer A:
(20mM TRIS, 0.1mM $CaCl_2$, 10mM ß-MCE, pH 7.5)
↓
Shake mixture end over end for 5min
↓
Add 0.3ml 5M NaCl
↓
Shake mixture end over end for 5min
↓
Add 0.2ml 0.1M $CaCl_2$
↓
Shake mixture end over end for 10min
↓
Pour into Column
(0.8cm x 4.0cm)
↓
Wash with 2ml Buffer A + 0.25M NaCl
↓
Wash with 2ml Buffer A
↓
Wash with 2ml Buffer A
↓
Elute Calpain II with 4ml 0.1M NaCl in Buffer B:
(20mM TRIS, 1mM EGTA, 10mM ß-MCE, pH 7.5)
↓
Elute Calpain I with 2ml Buffer B

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