final report

Project code: A.MQT.0013 Prepared

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Date submitted: June 2007

PUBLISHED BY
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Locked Bag 991
NORTH SYDNEY NSW 2059

Effects of pH, temperature and electrical inputs on muscle protein modifications that impact on meat eating quality

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government and contributions from the Australian Meat Processor Corporation to support the research and development detailed in this publication.

Abstract

This research has characterised the expression and post-translational modifications of proteins in muscle tissue that influence the commercial quality of fresh lamb. Variation in meat eating quality (tenderness) is largely attributed to the organisation of structural proteins within muscle and the activity of endogenous protease systems that remain active after rigor mortis. The addition or removal of functional groups from proteins, including phosphorylation, acylation, glycosylation, nitration, and ubiquitination, may alter the stability of the cell cytoskeleton. We are studying the phosphorylation patterns of proteins that influence cytoskeletal structure and proteolysis. Proteins with altered relative abundance or modification may provide targets for biomarkers of meat tenderness. Twenty lambs from similar genetic background were ranked for tenderness according to shear force (an objective measurement of the force required to bite through cooked meat). The four toughest and four most tender lambs were selected for phosphoproteome analysis.

Cellular fractions (connective tissue/myofibrillar and cytoskeletal) from muscle tissue were prepared by differential centrifugation and then subjected to affinity chromatography to enrich for phosphoproteins. The proteins are analysed by 2D electrophoresis to determine their relative abundance. Selectivity for phosphoproteins was verified by staining with the phosphoprotein stain Pro Diamond Q. The identity of the proteins and specific modifications of the proteins are subsequently characterised by mass spectrometry (MS) analysis including matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF/TOF) MS and liquid chromatography (LC) ion trap MS/MS. Current progress described here includes the identification of protein species and patterns of phosphorylation in muscle tissue that correlate with lamb tenderness. The biomarkers for predicting lamb tenderness determined in this experiment will be validated with industry partners to maximise the productivity and quality of Australian lamb products.

The public release of the fully annotated bovine genome sequence, which is due by 2006 will provide more information from which to search against and so potentially expand the list of identified markers within the tough and tender groups of *M. Longissimus dorsi* at rigour mortis.

Executive Summary

The objective of this research was to compare the protein expression of lamb loin samples taken at slaughter from similarly raised lambs at the extreme ends of the spectrum for tenderness from a sample of commercially produced and slaughtered lambs. The aim was to identify the significantly differing elements of the proteome between the tough and tender lamb samples at rigor.

Given the natural differences in subjective tenderness of meat derived from animals of similar environmental and genetic background, there is potentially large variation in the proteome of similar animals that contribute significantly to the tenderness of their meat. The tenderness of lamb differs most noticeably in the early stages of age conditioning (up to two days post-mortem) when large variability is evident in similar loin samples (Thompson *et al.*, 2004). This variability diminishes as proteolytic activity continues to degrade proteins in the myofibrillar structure as the meat ages. Common retailing practices deliver lamb to the consumer within two days of slaughter. This may have detrimental effects on the perceived sensory properties of the lamb if cooked and eaten prior to optimal age conditioning of the purchased cuts, particularly if the meat was from an animal of poor tenderness values at slaughter. Objective measurement of the tenderness of loin samples of lambs can be obtained nearing the conclusion of rigor through shear force testing of similarly cooked and treated loin samples. In this way, relatively tough and tender samples of lamb loins prior to the influence of any ageing or protein degradation can be obtained from animals of similar genetic and production backgrounds.

This “tough v tender” model for lamb samples collected at *rigor mortis* could be useful to provide evidence of important protein changes that relate to tenderness and also in identifying protein substrates and determining patterns of protein degradation that relate to shear force and meat tenderness.

The approach of this study was to sample lambs from similar genetic and environmental backgrounds and to determine those animals with divergent results for initial objective tenderness of their loin cuts.

This experiment was commenced in conjunction with a study of protein substrate degradation and proteolytic activity in meat being conducted by the MIRINZ research group of AgResearch in New Zealand. This group had developed a model for studying protein degradation and ageing in meat samples with the aim of understanding proteolysis and improving the sensory properties of meat for consumers.

Twenty lambs of this commercial consignment were stunned by captive bolt gun over a 20-minute period and identified with carcass tags. They were dressed conventionally and, prior to routine electrical stimulation, *m longissimus dorsi* (LD) samples from both sides of the carcass were taken at approximately 8-10min post-exsanguination. The LD samples were bagged and taken to the wet laboratory of MIRINZ (AgResearch) climate controlled to 15C.

The pH of the loins was monitored approximately every three hours through the decline to rigor (regarded as pH 5.6) using a Testo Model 230 (Germany) pH meter with glass electrode and temperature adjusting probe. A sample (~100g) of the LD was taken at the conclusion of rigor (pH5.6) and cooked for shear force testing

For the samples taken at rigor (pH 5.6), the shear force values were ranked in order for all 20 samples. The four toughest and four most tender samples were selected for protein expression analysis using proteomics techniques at the laboratories of Primary Industries Research Victoria, Werribee.

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1 Background

A national audit of retail lamb quality in Australia has shown that 20% of lamb sold for domestic consumption rated above the threshold for acceptable levels of toughness (Safari et al., 2002). These consumers place demands on the eating quality (particularly tenderness) of muscle when it is consumed as meat (SMART, 1994). However the reasons for large variation within and between animals in meat tenderness are not well understood. A large proportion of eating quality is determined by the structural integrity of muscle proteins. The ultimate nature of meat then, is a reflection of its protein deposition history and the changes that occur in these proteins up to slaughter (Oddy et al., 2002). Given the large range of natural variation in subjective tenderness of meat derived from animals of similar environmental and genetic background, there is great potential for changes in structural proteins in muscle to contribute significantly to the tenderness of meat.

Following exsanguination at slaughter, normal physiological regulation of biochemical processes in muscle cells is altered. Of particular relevance to meat quality are processes involved in provision of energy substrates, calcium homeostasis and proteolysis. These processes are intrinsically linked and are influenced by the biochemical history of the muscle up to slaughter and its post-slaughter temperature/pH environment. The tenderness of lamb differs most noticeably in the early stages of age-conditioning (up to two days post-mortem) when large variability is evident in similar loin samples (Taylor et al., 1995). This variability diminishes as proteolytic activity continues to degrade key cytoskeletal proteins in the myofibrillar structure as the meat ages (Taylor et al., 1995). However common retailing practices deliver lamb to the consumer within two days of slaughter. This may have detrimental effects on the perceived sensory properties of the lamb if cooked and eaten prior to optimal age conditioning of the purchased cuts, particularly if the meat was from an animal of poor tenderness values at slaughter.

Variation in meat tenderness can occur via three primary means, 1) differences in how muscle protein structures are built, through altered expression of structural proteins or structural protein isoforms during an animals growth (Crouse et al., 1991), 2) differences in the expression of proteins such as proteases that can modify the protein structures following slaughter (Koohmaraie, 1994), or 3) differences in the interaction between structural proteins and proteases as a result of changes in the biochemical environment of muscle following slaughter. However, post-translational modification of proteins is important in determining their susceptibility as substrates of proteases. There is increasing evidence to suggest that post-translational modification of cell structural proteins, including phosphorylation of cytoskeletal proteins, may be an important factor influencing the calpain mediated proteolytic degradation (Dulong et al., 2004; Schumacher et al., 1999). Hence, post-translational modification of proteins prior to slaughter may have important consequences for protein degradation during the post-mortem ageing period.

To date, meat quality research has failed to specifically and accurately describe the structural changes in muscle proteins that influence meat quality and variation in meat quality. Using a properly controlled study, whereby objective measurement of tenderness can be obtained nearing the conclusion of rigour through shear force testing of similarly cooked and treated loin samples, will enable investigation of how muscle protein expression and modifications can effect meat quality. In this way, relatively tough and tender muscle samples prior to the influence of any ageing or protein degradation can be obtained from animals of similar genetic and production backgrounds. Such studies will also allow identification of the cellular systems involved in production of quality meat.

2 Project Objectives

This project will use proteomics techniques to investigate the modifications (covalent and proteolytic) that occur in muscle proteins up to and following slaughter that influence meat quality. Characterising these changes will enable the identification of critical protein structures that specifically influence the eating quality of meat.

- Compare the protein expression profiles (proteome) of lamb loin samples taken at slaughter from similarly raised lambs at the extreme ends of the spectrum for tenderness from a consignment of commercially produced and slaughtered lambs;
- Characterised the proteins identified from objective 1 to provide a list of proteins whose structural integrity of abundance is related to tenderness/toughness;
- Identify post-translational modification of proteins associated with differences in initial tenderness/toughness;
- Validate these proteins from objectives 2 and 3 within the range of samples as potential biomarkers of toughness/tenderness;
- Provide a list of proteins and protein modifications that represent suitable biomarkers for tenderness/toughness to be validated further within the Australian red meat industry; and
- Provide information relating to technologies developed within this research for development of screening tools to better predict initial eating quality within the Australian red meat industry based on utilisation of the biomarkers discovered within this research.

3 Methodology

3.1 Muscle Tissue Collection and Initial Shear Force (Tenderness) Measurements

A consignment of twenty lambs were soured from an individual grower to provide a sample of lambs of similar genetic and production histories, likely to have divergent shear force values at rigor. The consignment was slaughtered at a commercial abattoir operated by Wilson Hellaby Foods (NZ) next to the Ruakura Research Station, Hamilton, NZ. The lambs were of mixed sex, of Romney Marsh breeding and estimated to be four months old with carcass weights in the range of 14 – 18 kg produced in the Waikato region of NZ.

Lambs were stunned by captive bolt gun over a 20-minute period and identified with carcass tags. They were dressed conventionally and prior to routine electrical stimulation, *m longissimus dorsi* (LD) samples from both sides of the carcass were taken at approximately 8-10 min post-exsanguination. The LD samples were bagged and taken to the wet laboratory of MIRINZ (AgResearch) which is climate controlled to 15°C. Pre-rigor samples (~5 g) were cut from the cranial end of one LD muscle from each lamb at approximately 35 minutes post-slaughter, then snap frozen in liquid nitrogen and stored at –80°C for subsequent proteomic analysis.

Initial pH measurements were taken for each loin and the bags were immersed in a 15°C water bath to undergo normal processes of rigor mortis and to ensure temperature differentials in the muscle did not influence rate of pH decline nor impact on natural proteolytic activities of the muscle. The pH of the loins was monitored approximately every three hours through the decline to rigor (regarded as pH 5.6) using a Testo Model 230 (Germany) pH meter with glass electrode and temperature adjusting probe. A sample (~100 g) of the LD was taken at the conclusion of rigor and cooked for shear force testing. The remainder of each loin was placed in cold storage at 4°C for sequential sampling and shear force testing at 12, 24, 48, 72 and 120 hours in order to regress shear force values against time and to develop a relationship for ageing rate for each lamb loin.

Shear force testing was conducted by cooking the denuded LD sample in a water bath maintained at 99°C to a mid-sample end point temperature of 75°C. Cooked samples were placed in an ice slurry until cool and sliced parallel to the muscle fibres to create about 10 rectangular prisms of 1cm^2 cross-sectional area. Each of these was placed in the MIRINZ Tenderometer and compressed to its yield point, with each measurement of force converted to kg shear force and averaged for the muscle sample.

3.2 Muscle Protein Extraction and Fractionation for Proteomic Analysis

Animals were ranked according to their initial shear force values taken at rigor. The four toughest and four most tender samples were selected for protein expression analysis. These samples were collected at approximately 35 min post-slaughter and maintained at –80°C. Prior to proteomic analysis the samples were fractionated to enable targeted analysis of muscle proteins from three different protein pools; i) extracellular matrix/myofibrillar proteins, ii) heavy structural proteins and iii) cytoskeletal/organelle proteins. Through our experience we have previously identified these protein pools to be those of interest and those to analyse.

Initially, a 2g LD muscle sample trimmed of fat was homogenised in 8ml of homogenisation buffer (40 mM Tris, 250 mM Sucrose, 5 mM $MgCl_2$, 2 mM EGTA, 10 mM KCl,

0.2% v/v Triton X-100, 10 mM DTT, Complete™ protease inhibitors) for 10 seconds using the lowest speed of an Ultraturrax 13mm homogenising probe. Samples were then subjected to differential centrifugation to fractionate the homogenate into the respective protein pools. Homogenates were passed through a 100 μm filter by pulse centrifugation at 1,000g. The solid material retained upon the filter was removed and briefly washed with buffer and pulse centrifuged again to remove any contaminating fat. 150 mg of this material was then resuspended in 750 μl of solubilisation buffer (7M urea, 2M thiourea, 1% CHAPS, 1% ASB-14, 10mM Tris, 10mM Acrylamide and 5mM Tributyl phosphine), labelled ***fraction 1*** and represents the ***extracellular matrix/myofibrillar proteins.***

The filtrate of the 100 μm filter from fraction 1 was collected and centrifuged at 1,500g for 10 mins. The resultant pellet was collected, resuspended in 400 μl of solubilisation buffer, labelled ***fraction 2*** and represents the ***heavy structural proteins*** of muscle cells.

The supernatant of fraction 2, was collected and centrifuged at 12,000g for 10 minutes to clarify and remove mitochondria and other organelles. The supernatant here was collected and re-spun to 100,000g in an ultra centrifuge for 30min and the pellet collected and solubilised in 200 μl of the solubilising buffer, labelled ***fraction 3*** and represents the ***cytoskeletal/organelle proteins***.

3.3 Analysis of Muscle Protein Fractions by 2 Dimensional Electrophoresis (2DE)

The protein content of each fraction from the 4 individuals with the highest and the 4 individuals with the lowest shear force at rigor were determined using the 2D Quant Kit prior to 2DE analysis. Any insoluble material was then sedimented by centrifugation at 100,000g for 1hr at 20°C. One hundred and fifty μg of protein from each fraction was then applied to 18 cm pH 3-10 IPG strips (Amersham Biosciences) in triplicate. Isoelectric focussing of IPG strips used the following protocol; 500V for 1hr, gradient to 1000V for 1hr, gradient to 8000V for 2 hrs and 8000V for 6 hours, for approximately 60,000 total volt hours. Focussed IPG strips were equilibrated for 15 minutes prior to application on 10-14% SDS polyacrylamide gels, which were subsequently run at 100V for 60 minutes, followed by 200V for 16 hours. Gels were then stained with SYPRO Ruby and the protein images captured using a cooled scanning CCD camera with excitation at 430nm, emission at 620nm and 4 seconds exposure. Scanning used a dynamic range of 0 to 65,000 grey levels and a resolution of 100μm.

Images from triplicate gels of each fraction were analysed to identify the two gels of highest reproducibility. Duplicate gels of each individual were then used for analysis of each fraction (8 gels/ extreme) to identify proteins with altered relative abundance between tender/tough extremes. Image analysis used Phoretix 2D Evolution software (Version 2002.01, Nonlinear Dynamics, UK). Proteins were detected and quantified using the Evolution Detection algorithm after the gel background was subtracted and the protein volume was normalised. Protein patterns of individual gels were warped to match each other to adjust for slight variations in relative spot position and pattern between gels. Duplicate gels for each individual were grouped together within tender/tough groups and an average gel for each of the tough and tender groups was produced. Only protein spots that were matched in 6 out of the 8 individual gels per group were represented on the average gel and used for statistical analysis. Changes in the relative abundance of proteins between tender/tough group were determined by the ANOVA function within Phoretix 2D Evolution. Differences in relative abundance of proteins were defined as significant where the normalised spot volumes between groups differed by $P < 0.05$.

3.4 Identification of Detected Proteins by MALDI MS/MS Analysis

Proteins that were detected with altered relative abundance between the tough and tender groups were excised from the 2DE gels using the Pro-Pic Investigator robot from Genomics Solutions. Individual gel plugs containing the protein samples were placed into sequential wells in a 96 well format plate, and subjected to two rounds of destaining with 25 mM ammonium bicarbonate and dehydration with 50% acetonitrile. The gel plugs were then fully dehydrated at 37 ºC prior to digestion with porcine trypsin for 16 hours at 37 ºC.

Digestion products were released from the gel plugs by sonication then analysed by tandem MALDI (Matrix Assisted Laser Desorption Ionisation) TOF/TOF mass spectrometry using the Bruker Ultraflex mass spectrometer. We utilised the Bruker Anchor chip technology that concentrates the digested peptide samples onto small hydrophilic targets thus improving the sensitivity of analysis. Each sample was applied to the Bruker Anchor Chip pre-coated with a thin layer of HCCA matrix, washed to remove any contaminating salts, allowed to dry and then analysed immediately. Samples were initially analysed in single MS mode to determine the peptide mass fingerprint of the sample. The three most intense peptide peaks were then ionized further and analysed in tandem MS/MS mode - which is able to determine the *de novo* amino acid sequence of the peptides selected. The single MS and tandem MS/MS data was then collated and combined to generate a Mascot generic file (.mgf) for each protein sample. These files were used by the DPI in-house Mascot bioinformatic search engine to search the NCBInr database (taxonomy: other mammalia) to identify the protein. Therefore, where data was available proteins were identified via their peptide mass fingerprint and deduced amino acid sequence determined by single MS and tandem MS/MS respectively. Protein identity was reported for samples that gave a significant ($P<0.05$) MOWSE (molecular weight search) score.

3.5 Post Translational Modification (PTM) Analysis of Muscle Proteins

PTM of a protein can influence the biological characteristics and properties of that protein. Therefore we wanted to investigate any PTM of the proteins present in the tough and tender animals, to determine if there were any differences between the groups.

3.5.1 Analysis of PTM Proteins by Specific Staining of 2DE Gels

The protein fractions isolated from the tough and tender grouped animals were subjected to 2DE analysis essentially as described previously (section 3.3). Proteins were separated by charge in the first dimension on longer 24 cm pH 3-11 non linear IPG strips (Amersham Biosciences) for 80 000 total volt hours. Then in the second dimension by size being subjected to electrophoresis through rhinohide strengthened 12 % polyacrylamide gels. To determine the proteins which have undergone PTMs (more specifically phosphorylation) the gels were then stained with Pro-Q® Diamond Phosphoprotein gel stain. Proteins within the gels were fixed overnight in 40% methanol, 10% acetic acid before being incubated in the stain for 3 hours. Images of the phosphorylated proteins were captured using a cooled scanning CCD camera with excitation at 540 nm, emission at 590 nm with a 30 second exposure. The phosphoprotein stain was removed by washing and then all the proteins stained with SYPRO Ruby. Total protein images were captured using the same camera with excitation at 460 nm and emission at 650 nm with a 2 second exposure. Differences in the phosphorylation of proteins between the tough and tender groups of fractionated samples were analysed as previously described.

3.5.2 Multi-Dimensional Liquid Chromatography Mass Spectrometry Analysis of Phosphorylated Proteins

Specific staining of the proteome of the tough and tender grouped lamb *M. longissimus dorsi* analysed by 2DE gels indicated that a number of proteins had undergone PTM's ie had been phosphorylated. Here we have advanced this research by affinity purifying these modified proteins and identifying them through multi-dimensional liquid chromatography mass spectrometry (LCMS).

Fraction 1 (extracellular matrix/myofibrillar) proteins and fraction 3 (cytoskeletal/organelle) proteins were used within this analysis as these pools contained the protein markers of interest. Protein samples were precipitated by adding 10 volumes of ice cold acetone, collected by centrifugation and resuspended in the lysis buffer supplied with the PhosphoProtein purification kit. Samples were applied to the phosphoprotein affinity columns which specifically bind the phosphorylated proteins only. These proteins were then eluted from the column, desalted through a PD-10 column and finally concentrated by acetone precipitation a second time. Samples were resuspended in solubilisation buffer, reduced and alkylated with TBP and acrylamide prior to digestion with trypsin. 10 μg of the digested phosphoproteins were then analysed by LCMS. We used the EttanTM MDLC, an automated micro- and nanoflow multidimensional HPLC, connected to a Thermo Finnigan LTQ LC MS/MS system located at PIRVic Werribee. Peptide samples were bound to a reverse phase C-18 column and eluted with an acetonitrile gradient in to the Ion Trap MS/MS analyser. Mass spectrometric data generated was analysed using the Finnigan Xcalibur Bioworks 3.2 software. Protein identification was achieved in the software with a TurboSEQUEST search against a bovine FASTA database.

In order to characterise the PTM's further those peptides that were phosphorylated were specifically enriched for. The phosphorylated peptides within the tryptic digests were affinity purifed by binding to titanium dioxide (TiO_2) tips, thus removing any non-phosphorylated peptides from the digests. The bound phosphorylated peptides were washed, then eluted from the tips and analysed on the same LCMS system. The MS data was searched against the latest ovine and bovine databases from Uniprot Consortium using the Bioworks 3.3 software, employing stringent filters (peptide score<E-03) to determine the specific peptides were phosphorylated.

3.6 Validation of Initial Toughness/Tenderness Protein Markers by Western Blot Analysis

Potential biomarkers for initial toughness/tenderness of lamb samples were assessed by Western blot analysis of all 20 lamb samples. Each muscle sample (1 g frozen weight) was homogenised in 7.5 ml homogenisation buffer (40mM Tris, 250mM Sucrose, 5mM MgCl2, 2mM EGTA, 10 mM KCl, 0.02% Triton X-100) containing nuclease and protease inhibitors, prior to incubation on ice for 30 mins. 2.5 ml of 4X SDS PAGE sample buffer was added to each sample which was then heated to 95°C for 10 mins to fully dissolve the proteins. Any proteins that did not resuspend were sedimented and removed by centrifugation.

Aliquots of each sample were then subjected to 1D electrophoresis through a 4-12% gradient mini gel and transferred to PVDF membrane (20 V for 20 mins) for Western blot analysis. The membranes were first blocked with 5% skim milk and then probed with the relevant primary antibody, followed by the HRP conjugated second antibody. The protein markers analysed were Calsarcin/Myozenin, Troponin T, Tropomysin 4, Annexin II, Proteasome 20S X

and Y, Sarcoglycan and Calsequestrin. The exact antibody concentrations required for each individual analysis were determined prior to analysis against representative samples. Protein levels of the biomarkers were then detected by chemileminescent using an ECL kit against film. Biomarker levels were measured by scanning the images and correlated against shear force values for each lamb loin muscle sample in order to verify the biomarkers.

4 Results and Discussion

4.1 Tenderness Measurements of Samples.

M longissimus dorsi (LD) muscle was removed from each of the 20 commercially raised and slaughtered lambs. Shear force (tenderness) measurements were conducted at rigor and at 12, 24, 48 and 120 hrs thereafter (table 1). The animals were ranked according to their initial shear force measurements taken at rigor; the 4 toughest animals were # 1,7,14 and 15 (highlighted in red) and the 4 most tender were # 2,8,10 and 17 (blue). These 8 animals were selected to represent the initial tough and tender groups of animals respectively.

Animal Number	Shear Force Measurements (kg)				
	Rigor	12 Hr	24 Hr	48 Hr	120 Hr
1	**19.0**	**14.6**	**9.4**	**5.4**	**4.6**
2	13.1	8.0	5.4	4.3	3.7
3	16.2	8.8	5.7	4.9	3.6
4	15.7	14.9	15.9	9.0	5.7
5	18.2	11.3	10.4	5.1	4.3
6	15.1	10.0	4.9	5.4	4.1
7	**18.6**	**12.8**	**12.5**	**5.3**	**4.2**
8	**14.2**	**7.6**	**7.8**	**4.0**	**3.6**
9	17.1	9.9	8.7	4.8	5.0
10	**13.3**	**9.0**	**4.7**	**4.3**	**4.5**
11	16.0	13.1	9.9	5.5	5.7
12	16.4	9.0	5.4	5.0	4.8
13	14.7	12.7	6.8	3.7	4.1
14	**18.8**	**8.4**	**4.9**	**4.6**	**5.0**
15	**18.2**	**16.2**	**14.5**	**6.5**	**7.6**
16	17.8	11.3	5.7	4.1	4.3
17	**13.1**	**11.0**	**7.8**	**4.1**	**5.2**
18	16.4	11.3	8.6	6.8	7.4
19	14.2	9.8	8.2	5.2	4.4
20	15.1	11.6	9.6	5.1	5.7

Table 1: Shear Force Measurements

The initial toughness and tenderness characteristics remained for the first 48 hours of the aging process for the majority of these animals, with those initially tough producing a higher shear force than the initially tender animals (figure 1). However at 120 hours post rigor the shear force values were similar for all animals, indicating that the aging process was complete.

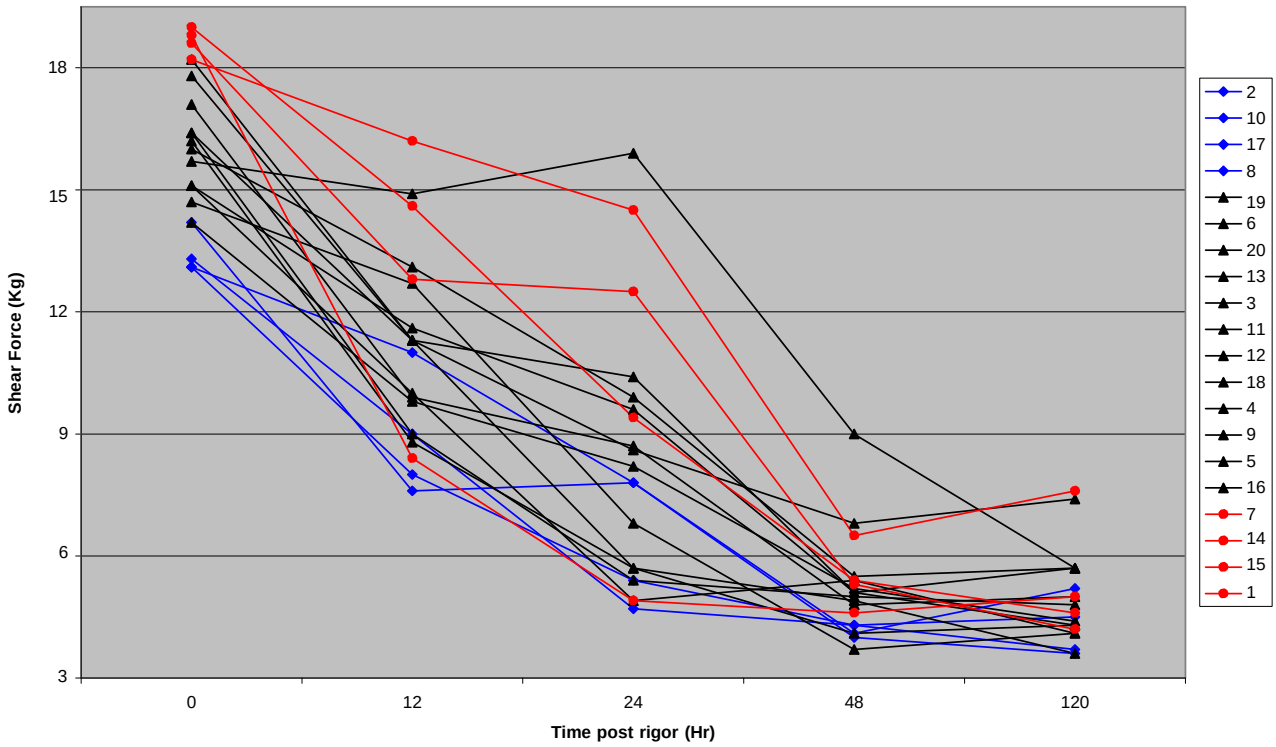


Figure 1: Aging rates of the muscle samples. *Shear force values were determined for each lamb sample at rigor then at 12, 24, 48 and 120 hr post rigor and plotted against time. The 4 initial toughest animals are shown in red and the 4 most initial tender in blue.*

4.2 Proteomic Analysis of Protein Markers for Initial Toughness and Tenderness.

To identify potential protein markers of initial toughness and tenderness the proteome of the tough and tender animals was analysed by 2DE. Proteins from each of the 8 animals were isolated and then further fractionated into the extracellular matrix/myofibrillar proteins, heavy structural proteins and cytoskeletal/organelle proteins prior to 2DE. Fractions were analysed in triplicate to determine those proteins with altered levels between the tough and tender samples and may be biomarkers.

Thirty proteins were detected with altered relative abundance between the tough and tender groups within the extracellular matrix/myofibrillar fraction, (Figure 2; appendix Table a). Twenty five of these proteins showed significant differences in abundance between the two groups, with an additional 2 proteins identified only in the tough group and 3 proteins identified only in the tender group. The position of proteins from Fraction 1 with higher relative abundance in the tender group are circled in blue and proteins with higher relative abundance in the tough group are circled in red.

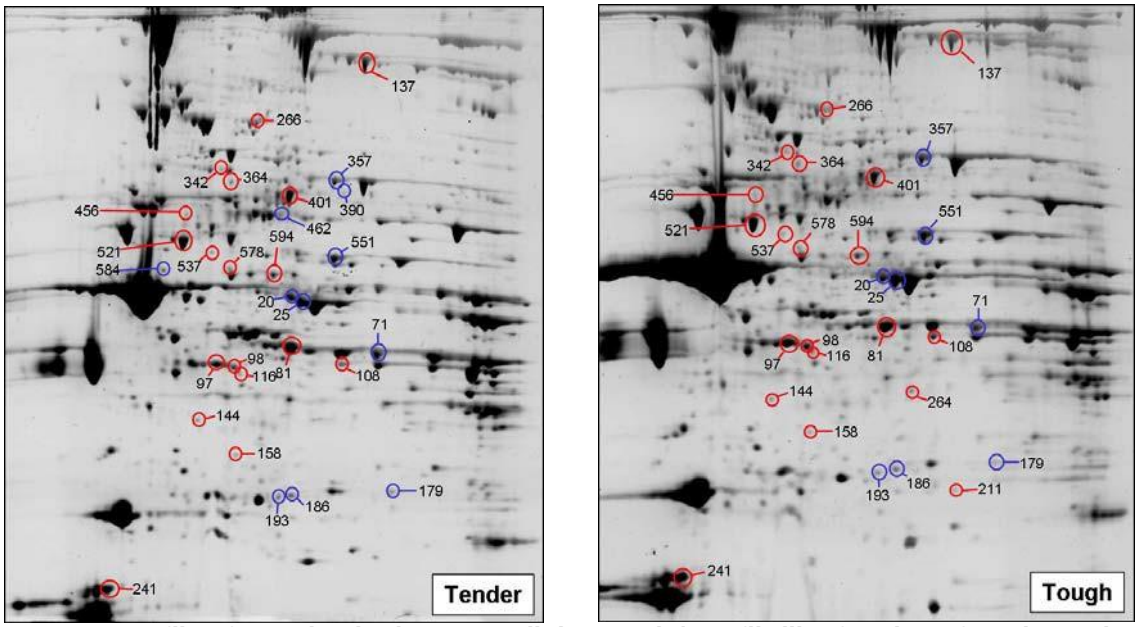


Figure 2. 2DE profile of proteins in the extracellular matrix/myofibrillar fraction of tender and tough lamb M. longissimus dorsi*. Proteins were separated between pH 3-10 in the first dimension and resolved by molecular weight in the second dimension by SDS PAGE using a 10-14% acrylamide gradient. Proteins with altered relative abundance are numbered and refer to those proteins listed in appendix Table a. Proteins circled in red have a higher relative abundance in the tough samples and those in blue have higher relative abundance within the tender lamb M. longissimus dorsi respectively.*

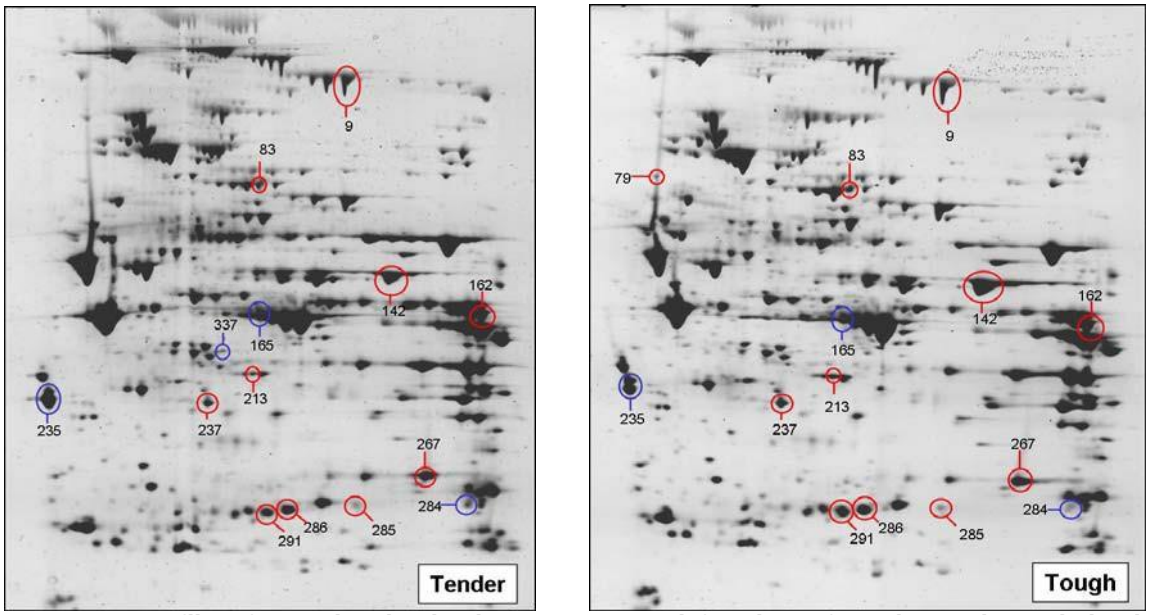


Figure 3. 2DE profile of proteins in the heavy structural fraction of tender and tough lamb M. longissimus dorsi*. Proteins were separated between pH 3-10 in the first dimension and resolved by molecular weight in the second dimension by SDS PAGE using a 10-14% acrylamide gradient. Proteins with altered relative abundance are numbered and refer to those proteins listed in appendix Table b. Proteins circled in red have a higher relative abundance in the tough samples and those in blue have higher relative abundance within the tender lamb M. longissimus dorsi respectively.*

For the heavy structural fraction, 16 proteins were detected with altered relative abundance between the tough and tender groups (Figure 3; appendix Table b). Fourteen of these proteins showed significant differences in abundance between the two groups, with an additional 1 protein identified only in the tough group and 1 protein identified only in the tender group. Within the cytoskeletal/organelle fraction, 34 proteins were detected with altered relative abundance between the tough and tender groups (Figure 4; appendix Table c). Twenty eight of these proteins showed significantly higher relative abundance in the tender group, with 6 of these proteins showing significantly higher relative abundance in the tough group.

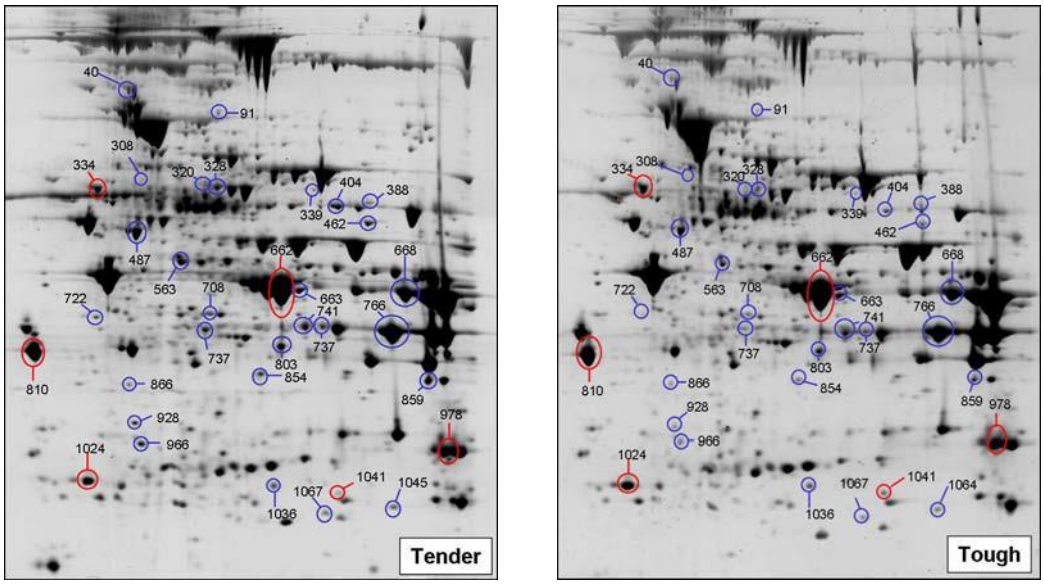


Figure 4. 2DE profile of proteins in the cytoskeletal/organelle fraction of tender and tough lamb M. longissimus dorsi. *Proteins were separated between pH 3-10 in the first dimension and resolved by molecular weight in the second dimension by SDS PAGE using a 10-14% acrylamide gradient. Proteins with altered relative abundance are numbered and refer to those proteins listed in appendix Table c. Proteins circled in red have a higher relative abundance in the tough samples and those in blue have higher relative abundance within the tender lamb M. longissimus dorsi respectively.*

In total 80 proteins were detected to have differential abundance between the tough and tender lamb LD muscle samples at rigor. In order to identify these proteins they were then excised from the 2D gels and analysed by tandem MALDI MS. By searching the MS/MS data against databases we were able to identify 55 of these proteins from the 3 protein fractions (Tables 2-4). We would not expect all of the 55 proteins identified to be directly related to the mechanism responsible for variation in initial toughness/tenderness. Therefore we refined this list of potential targets by selecting proteins that are of interest for maintaining structural integrity of muscle cells. Such target proteins would include members of the extracellular matrix and transmembrane/adhesion molecules, the intracellular cytoskeletal proteins and proteins involved in the organisation of the contractile filament structure. Structural proteins were of primary interest, as their relative amounts in muscle may be indicative of the development and stability of intra and extracellular structures. Further to this, the degradation of structural proteins by proteases is responsible for changes in meat tenderness post-mortem; therefore proteases were included as well. A number of highly interesting proteins were selected that could be potential markers and their location within the 2DE profiles are highlighted in Figure 5.

Table 2. List of extracellular matrix/myofibrillar proteins with altered relative abundance between tough and tender lamb M. longissimus dorsi at rigor. *Spot numbers refer to proteins identified in Figure 2.*

	Normalised volume		SEM	P value	Protein ID
	Tender	Tough			
357	0.821	0.509	.120	.049	**pyruvate kinase 3 isoform 2**
401	0.894	1.169	.112	.043	**dihydrolipoamide dehydrogenase precursor**
551	1.283	0.754	.173	.042	**enolase 1**
137	0.644	0.947	.096	.031	**aconitase 2, mitochondrial**
594	0.292	0.396	.031	.023	**Tu translation elongation factor, mitochondrial**
364	0.065	0.111	.012	.022	**hypothetical protein LOC506238**
521	1.243	1.528	.094	.016	**ubiquinol-cytochrome c reductase core protein I**
266	0.121	0.277	.049	.011	**succinate dehydrogenase flavoprotein subunit A**
456	0.027	0.054	.008	.008	**sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein)**
537	0.029	0.062	.005	<.001	**proteasome (prosome, macropain) 26S subunit, ATPase 2**
584	0.699	-	.057	-	**eukaryotic translation initiation factor 4A2**
81	0.591	0.722	.045	.041	**troponin T3, skeletal, fast**
97	0.487	0.782	.088	.039	**troponin T1, skeletal, slow**
116	0.030	0.074	.016	.034	**lactate dehydrogenase B**
193	0.086	0.056	.011	.033	**triosephosphate isomerase**
25	0.813	0.566	.079	.021	**creatine kinase, muscle**
98	0.167	0.253	.028	.029	**troponin T1, skeletal, slow**
20	0.417	0.221	.059	.010	**creatine kinase, muscle**
241	0.420	0.711	.085	.009	**myosin light chain 2**
186	0.123	0.078	.013	.007	**triosephosphate isomerase**
264	-	0.039	.008	-	**myozenin 1**

Table 3. List of heavy structural fraction proteins with altered relative abundance between tough and tender lamb M. longissimus dorsi at rigor. *Spot numbers refer to proteins identified in Figure 3.*

Spot number	Normalised volume		SEM*	P value	Protein ID
	Tender	Tough			
9	0.643	1.030	.140	.046	**aconitase 2, mitochondrial**
165	0.406	0.270	.059	.042	**creatine kinase, muscle**
237	0.206	0.293	.032	.041	**cytosolic malate dehydrogenase**
213	0.406	0.270	.059	.041	**similar to Calcium/calmodulin-dependent protein kinase type IV ***
291	0.405	0.551	.044	.023	**triosephosphate isomerase**
285	0.096	0.159	.017	.023	**KIAA1833 protein, partial**
286	0.522	0.676	.030	.018	**triosephosphate isomerase**
142	1.273	2.286	.226	.007	**Beta enolase (2-phospho-D-glycerate hydro-lyase) (Muscle-specific enolase)**
162	1.090	1.566	.131	.007	**phosphoglycerate kinase 1, partial**
267	0.531	1.075	.090	.001	**carbonic anhydrase-like protein**

Table 4. List of cytoskeletal/organelle fraction proteins with altered relative abundance between tough and tender lamb M. longissimus dorsi at rigor. *Spot numbers refer to proteins identified in Figure 4.*

Spot Number	Normalised volume		SEM*	P value	Protein ID
	Tender	Tough			
1036	0.088	0.073	.006	.049	**similar to Proteasome subunit alpha type 2 (Proteasome component C3) (Macropain subunit**
663	0.222	0.200	.008	.049	**aminotransferase 1] [glutamic-oxaloacetic transaminase 1, soluble]**
462	0.092	0.050	.013	.049	**NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa**
328	0.105	0.053	.023	.046	**dihydrolipoamide dehydrogenase**
487	0.342	0.236	.038	.046	**ubiquinol-cytochrome c reductase core protein I**
766	2.204	1.764	.145	.046	**Calpastatin type 1 * / GAPDH**
1041	0.038	0.061	.008	.042	**glutathione S-transferase pi**
563	0.174	0.105	.027	.040	**NADH:ubiquinone oxidoreductase (428 AA**
388	0.041	0.022	.007	.034	**ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit**
1067	0.051	0.032	.006	.029	**vacuolar protein sorting 33A, partial**
320	0.053	0.024	.011	.029	**TXNRD3 protein, partial**
1064	0.067	0.050	.007	.027	**proteasome beta 5 subunit**
668	1.385	0.964	.150	.026	**fructose-1,6-bisphosphate aldolase A, partial**
866	0.035	0.026	.003	.025	**annexin A4**
859	0.152	0.099	.019	.024	**voltage-dependent anion channel 1**
966	0.069	0.061	.010	.023	**NADH dehydrogenase (ubiquinone)**
662	4.031	4.824	.263	.017	**creatine kinase, muscle**
978	0.687	0.827	.052	.016	**PARK2 co-regulated, partial**
737 =737B	0.125	0.067	.015	.012	**Skeletal muscle-specific calpain (Fragment)**
404	0.132	0.064	.016	.010	**ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit**
334	0.283	0.424	.036	.009	**Alpha-tubulin 1 (Fragment)**
757 = 737A	0.251	0.045	.065	.007	**Enolase (Fragment)**
810	1.370	2.278	.222	.005	**similar to tropomyosin 4, partial**
803	0.300	0.202	.026	.004	**annexin A2**

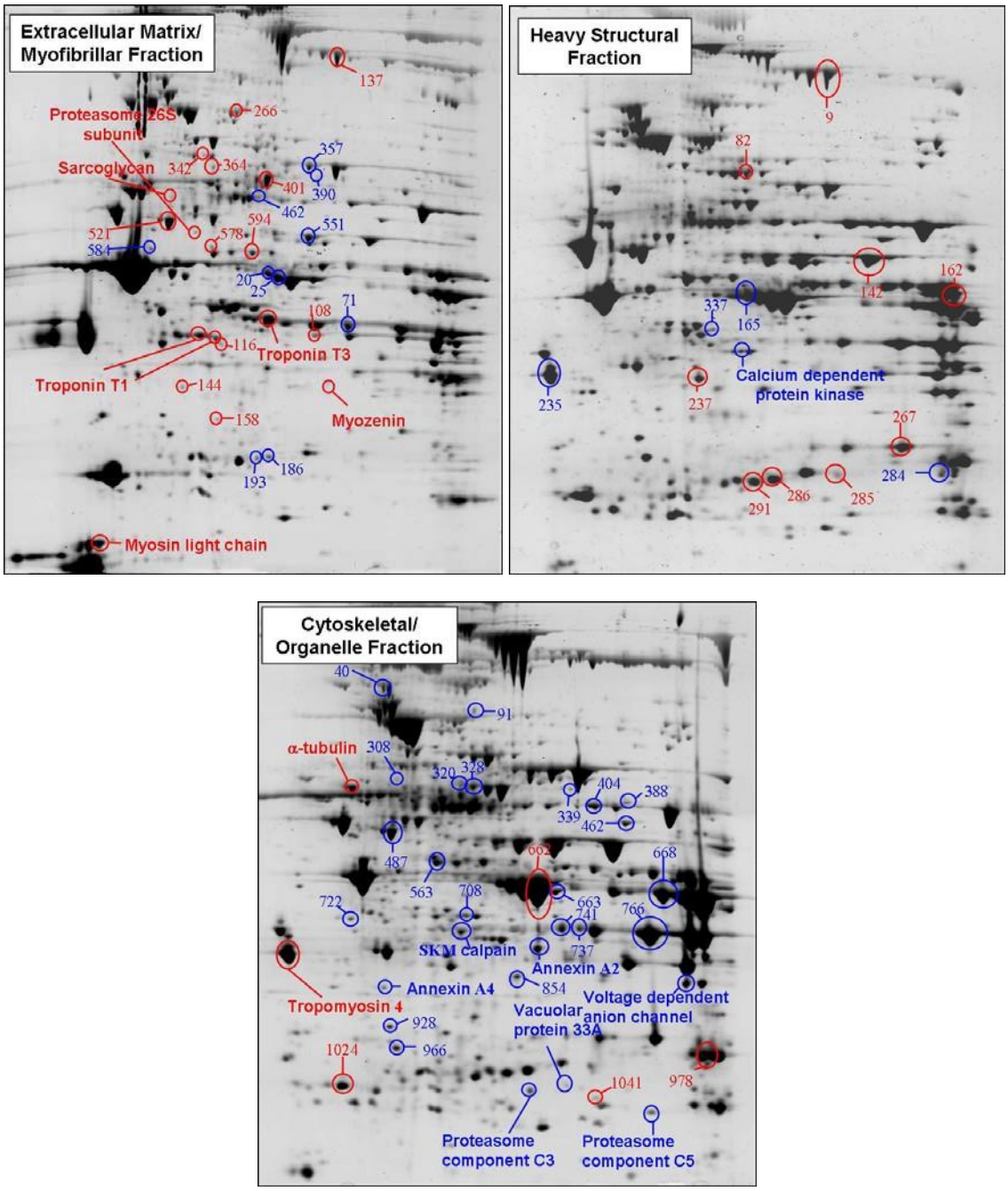


Figure 5. 2DE profile of identified proteins of high interest with altered relative abundance in the respective fractions of tender and tough lamb M. longissimus dorsi*. Proteins shown in red have a higher relative abundance in the tough samples and those in blue have higher relative abundance within the tender lamb.*

Within the list of potential markers for the prediction of initial toughness and tenderness a number of structural proteins were identified with higher relative abundance in the tough group. Sarcoglycan, myozenin1, troponin T1 and T3, myosin light chain, α-tubulin and tropomycin-4 were all elevated within the tough samples and so potentially contribute to the toughness of these samples. Increased abundance of tropomysin 4, troponins T1 and T3 are consistent with expected changes relating to initial toughness within the literature. Interestingly, increased abundance of sarcoglycan in tough meat suggests that the cytoskeletal/sarcolemmal dystrophin complex that integrates myofibrils, the sarcolemma and the extracellular matrix may be important in determination of initial toughness. Sarcoglycan dysfunction has been shown to influence force transduction and is involved in some muscular dystrophies. Myozenin is a skeletal muscle Z-line actin-associated protein that may also be important in muscular dystrophies. Elevated myosin light chain and α-tubulin suggest that degradation of major myofilament contractile and cytoskeletal structural proteins may be more crucial to determination of tenderness than previously accepted in the literature.

The voltage dependent anion channel protein and annexin A2 and A4 were present with higher abundance in tender samples indicating enhanced trafficking of proteins. Annexins are calcium-binding proteins that may be associated with cross-linking the membrane and cytoskeletal proteins. This and elevated voltage dependent anion channel protein suggests altered ionic flux mechanisms operate within meat of initial high tenderness. Three protein families whose functions are associated with proteolysis; calpain, proteosome and the vacuolar protein (localised in lysosomes) were all elevated in the tender group, indicating roles for these three systems in determination of tenderness. Of particular note is the identification of proteasome subunits in tender meat, indicating a more prevalent role for the proteasome in degradation of proteins associated with tenderness. Identification of elevated skeletal muscle specific calpain in tender meat is an exciting finding considering its unproven putative role in muscle protein turnover.

Proteolysis is fundamental to the tenderisation process and within the tender group of samples we observed a reduction in structural proteins and elevated proteases. We also noted that a calcium dependent protein kinase was upregulated in tender meat, this protein is able to modify the phosphorylation state of proteins. Post-translational modification of proteins is important in determining their susceptibility as substrates of proteases. There is increasing evidence to suggest that post-translational modification of cell structural proteins, including phosphorylation of cytoskeletal proteins, may be an important factor influencing their susceptibility to proteolytic degradation. Therefore, proteins such as kinases may have major roles in tenderisation by converting proteins into substrates for proteases. This will be investigated further by analysing PTMs of proteins within the tough and tender proteomes.

4.3 Analysis of PTM of Muscle Proteins within the Initially Tough and Tender Group of Animals.

To investigate if any proteins within the tough and tender muscle proteomes had undergone PTM or more specifically phosphorylation the proteomes were analysed by two proteomic approaches to profile the significantly differing elements between the tough and tender lamb samples at *rigor mortis*.

4.3.1 Specific Staining of Phosphorylated Proteins in 2DE Gels

Initially to determine if any proteins were phosphorylated the samples were re-analysed by our improved 2DE protocol and gels were first stained with Pro-Q® Diamond Phosphoprotein gel stain followed by SYPRO Ruby to then detect total protein present (Figure 6.). The new protocol employed to generate the 2DE protein maps enabled better separation of the proteins in the first dimension through the use of longer IPG strips. In addition the protein spots have a rounder more uniform shape with the Rhinohide gel strengthener compared the “arrow-head” appearance of the protein spots in the previous gels. However the protein profiles of both sets of gels were very similar. Staining of the gels with Pro-Q® Diamond demonstrated that a number of proteins were phosphorylated within both the tender and tough groups, and the differences between the groups are highlighted in blue and red on the gels.

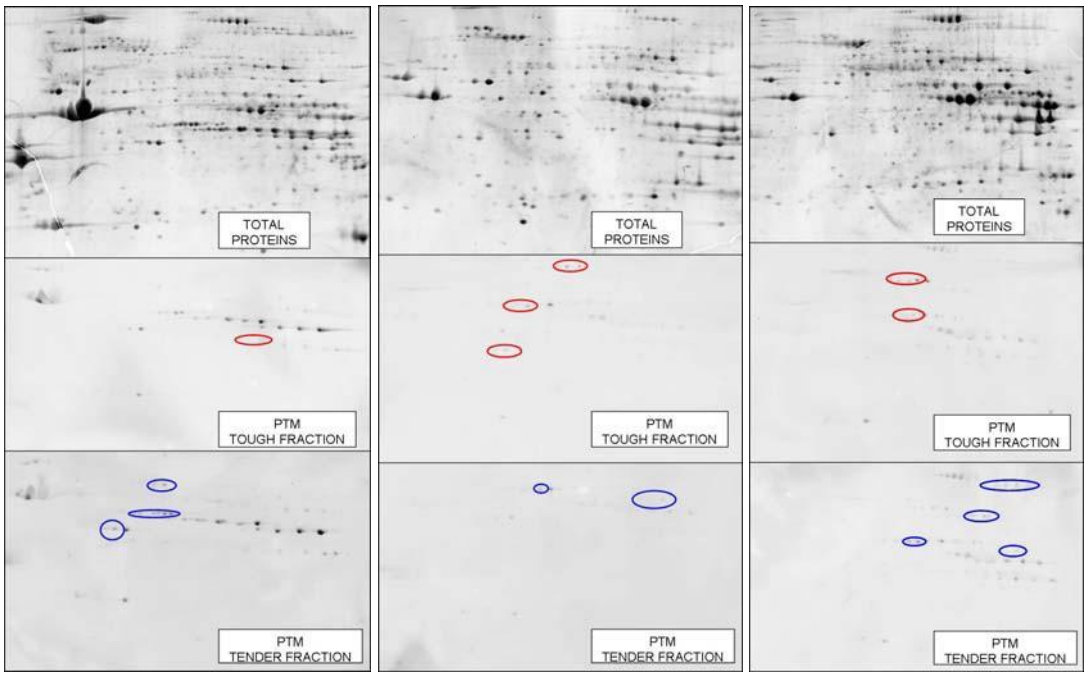


Figure 6. 2DE profile of proteins fractions of tender and tough lamb M. longissimus dorsi. *Proteins were stained with Sypro Ruby (top gels) and the phospho- specific Pro-Q® Diamond Phosphoprotein gel stain*

(lower gels). The three vertical panels represent the extracellular matrix/myofibrillar, heavy structural and ytoskeletal/organelle protein fractions respectively (left to right). Proteins shown in red have a higher relative abundance in the tough samples and those in blue have higher relative abundance within the tender lamb samples.

4.3.2 Analysis of Phosphorylated Proteins by LCMS

To enhance the identification of proteins we used a PhosphoProtein purification kit to isolate the phosphorylated proteins from the protein samples prior to analysis. To verify that this kit specifically binds phosphorylated proteins we applied a total muscle homogenate from a lamb sample of average initial toughness/tenderness to the column. Bound proteins (phosphorylated) and the unbound proteins (non-phosphorylated) were analysed by 2DE gels stained with SYPRO ruby for total protein analysis and Pro-Q® Diamond stain to analyse phosphorylated proteins. Gel images (Figure 7a) indicated that of the total proteins present in the homogenate (left panel) only a very low percentage were phosphorylated (right panel). As expected when this extract was applied to the PhosphoProtein column the majority of the proteins did not bind to the column (Figure 7b – left panel) and of these very few were phosphorylated (right panel). However analysis of the proteins which bound to the column (Figure 7c) showed that these interacted with both the SYPRO (left panel) and Pro-Q® Diamond stains (right panel) indicating that these proteins were phosphorylated. Therefore this column highly enriches for and purifies the phosphorylated proteins and confirms that the PhosphoProtein purification kit is a very efficient means of purifying the phosphorylated proteins thus enabling their analysis within this study.

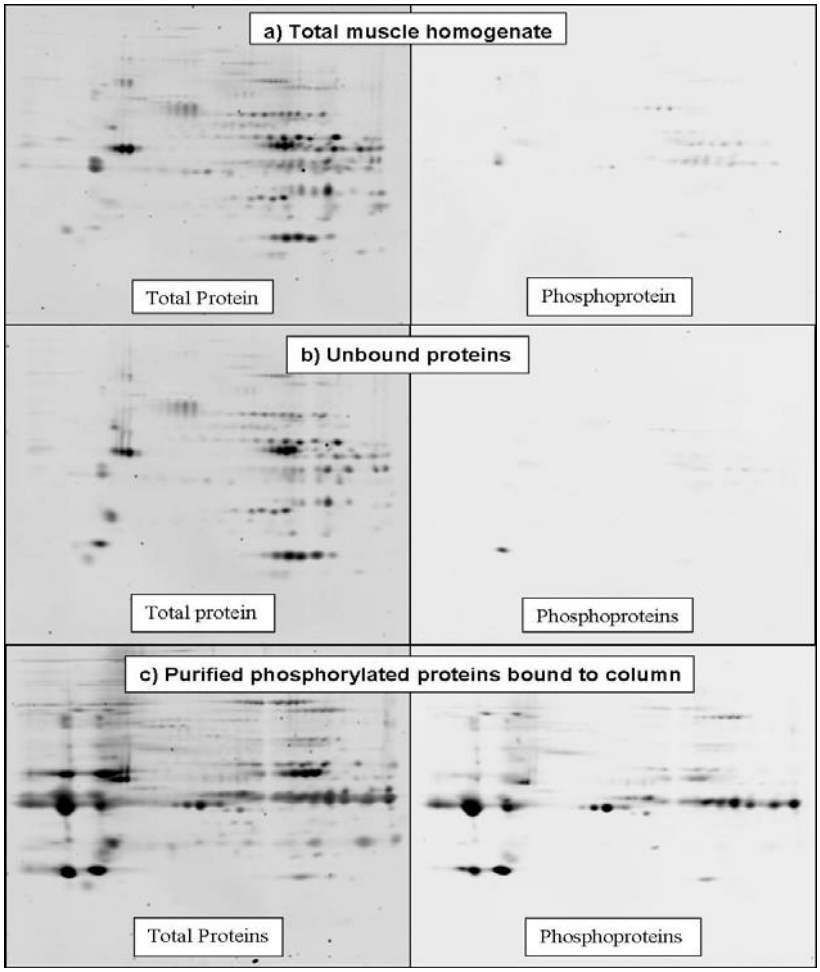


***Figure 7**: **2DE analysis of the purification of phosphorylated proteins from a lamb muscle extract**. The total homogenate **a)** was applied to the phosphoprotein column, those proteins which did not bind **b)** and those purified by binding to the column **c)** were analysed on the gels. All proteins were visualised with SYPRO ruby stain in the (left) and the phosphorylated proteins were specifically stained with Pro-Q® Diamond stain (right).*

Therefore we used the kit to specifically isolate the phosphorylated proteins from the extracellular matrix/myofibrillar and cytoskeletal/organelle protein fractions isolated from the 4 extreme tender and tough muscle samples. These protein fractions were selected as all the structural protein biomarkers identified previously were derived from these protein fractions. The purified phosphoproteins were visualised on 2DE gels stained with SYPRO ruby (Figure 8). These gels indicated that there were differences between the phospho-proteome of the tough and tender samples. However as only about 10% of proteins are phosphorylated it was not possible to identify the phosphoproteins by MALDI TOF/TOF analysis. Therefore we utilised the more sensitive multi-dimensional LCMS system.

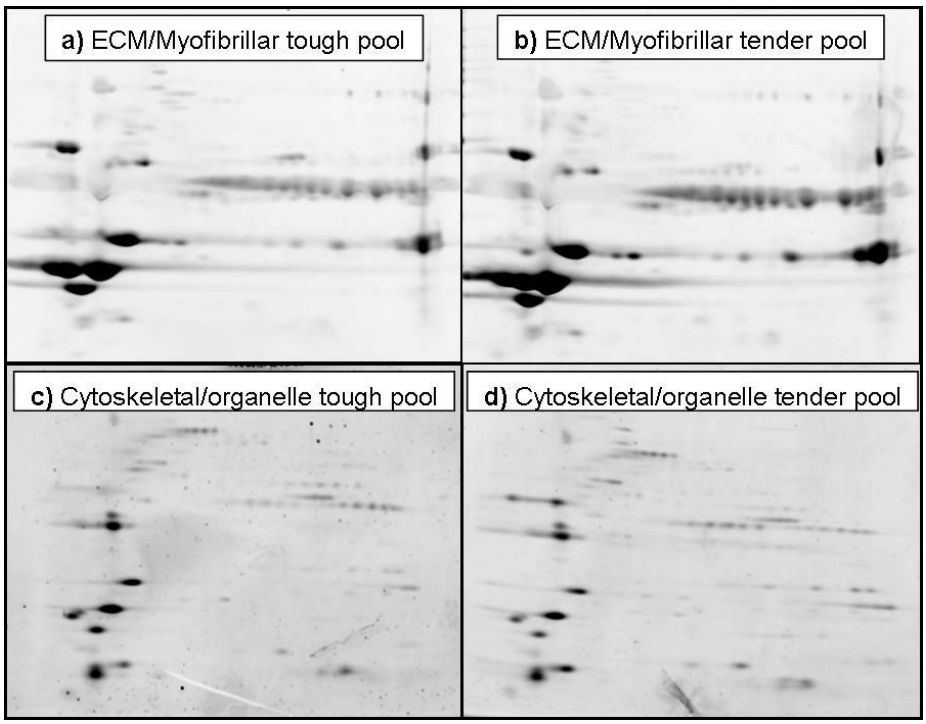


***Figure 8**: **2DE analysis of purified phosphorylated proteins from lamb muscle protein fractions.** Phosphorylated proteins were purified from the extracellular matrix/myofibrillar and cytoskeletal/organelle protein fractions obtained from the 4 most tender and tough muscle samples using the PhosphoProtein column. Pooled samples were subjected to 2DE analysis and the gels stained with SYPRO ruby. The gels represent the phosphoproteins present in the extracellular matrix/myofibrillar fractions from tough **(a)** and tender **(b)** pools and the cytoskeletal/organelle fraction pooled from tough **(c)** and tender **(d)** animals.*

To obtain confidence in the identification of the proteins we have applied stringency filters of Pep (peptide) scores less than P<0.01, or Prot (protein) correlation scores of greater than 20. This enables significant identification of proteins from peptide sequence or peptide mass data (or both). Where Pep and Prot scores are given for both the tough and tender groups, the protein was identified in at least 2 individuals of the tough group and the tender group. Where Pep and Prot scores are given for the tough group or the tender group only, the protein was not identified in both groups and represents a difference in post-translational modification particular to tough or tender animals.

We identified 128 phosphorylated proteins within the extracellular matrix/myofibrillar (Appendix Table d) and cytoskeletal/organelle (Appendix Table e) protein fraction samples that satisfied our stringent filters for ID confidence. Of these 10 were phosphorylated specifically in muscle from tender animals and 7 from tough animals. Within the extracellular matrix/myfibrillar fraction, a 70 kDa heat shock protein, GTP-binding regulatory protein, monocyte chemotactic protein, cartilage derived morphogenic protein, retinol binding protein, diazepam binding inhibitor and a lipooxygenase were all phosphorylated specifically in muscle from tender animals. The potassium channel, P57 protein, collagen alpha 1 and nicotinic acetylcholine receptor delta chain were phosphorylated specifically in muscle from tough animals. Analysis of the

cytoskeletal/organelle fraction indicated a 10 kDa heat shock protein, a P64 chloride channel protein and an unc-18 homolog protein were all phosphorylated specifically in muscle from tough animals. Additionally a polar organelle development protein, NADH2 dehydrogenase and myosin heavy chain beta were phosphorylated specifically in muscle from tender animals. However of these only 2; Collagen 1 α and myosin heavy chain β are structural proteins. Of high interest was that myosin was specifically phosphorylated in muscle from tender animals. Myosin was previously identified as a biomarker with elevated levels in tough samples compared to tender. This may suggest that PTM of myosin alters it levels in muscle samples.

To further characterise the PTM, the specific peptides that were phosphorylated were purified on TiO_2 tips prior to LCMS analysis. Searching the Uniprot ovine and bovine databases identified a number of proteins that were specifically phosphorylated only in the tough or tender animals (Table 5). Three structural proteins were identified; Tensin was specifically phosphorylated in fraction 1 of tough animals, whereas in fraction 3 Troponin T was specifically phosphorylated in the tender animals and myosin light chain in the tough. This would suggest that the PTM of cytoskeletal myosin is critical to the toughness of muscle. If the heavy chain is phosphorylated the sample is tender and if the light chain is phosphorylated tough, indicating that specific modification of myosin chains characterise its susceptibility to proteolysis. In addition the phosphorylation of Troponin T influences the level of this protein within muscle. Tensin is a 200kDa protein that binds actin and is concentrated at focal adhesions and may link the cell membrane to the cytoskeleton (Lo et al 1994).

Table 5 Phosphorylated peptides identified in fraction 1 (a) and 3 (b) by LCMS following TiO_2 purification.

***a) extracellular matrix/myofibrillar proteins*.**

	Phosphorylated Protein	Phosphorylated Peptide Sequence
Tough	Myosin light chain 1, skeletal muscle isoform	K.DQGTYEDFVEGLR.V
Tender	Myosin light chain 1, skeletal muscle isoform	K.ITLSQVGDVLR.A
T&T	Myosin light chain 1, skeletal muscle isoform	K.KPAAAAAPAPAPAPAPAPAPPKEEK.I
T&T	Myosin light chain 1, skeletal muscle isoform	R.ALGTNPTNAEVKK.V
T&T	Myosin light chain 1, skeletal muscle isoform	R.HVLATLGEK.M
Tender	Myosin light chain 1, skeletal muscle isoform	R.VFDKEGNGTVMGAELR.H
T&T	Myosin regulatory light chain 2, skeletal muscle	K.EAFTVIDQNR.D
T&T	Myosin regulatory light chain 2, skeletal muscle	K.KFLEELLTTQCDR.F
T&T	Myosin regulatory light chain 2, skeletal muscle	K.LKGADPEDVITGAFK.V
T&T	Myosin regulatory light chain 2, skeletal muscle	K.NICYVITHGDAKDQE.-
Tough	Myosin regulatory light chain 2, skeletal muscle	K.NMWAAFPPDVGGNVDYK.N
T&T	Myosin regulatory light chain 2, skeletal muscle	R.DGIIDKEDLR.D
Tender	Myosin regulatory light chain 2, skeletal muscle	R.DTFAAMGR.L
T&T	Myosin regulatory light chain 2, skeletal muscle	R.LNVKNEELDAMMK.E
Tough	Myosin-1	K.EKS*EMKMEIDDLASNMETVSK.A
Tender	Myosin-1	K.NDLQLQVQSEADALADAEER.C
Tender	Myosin-1	K.YEETHAELEASQK.E
Tough	Myosin-1	R.DLEEATLQHEATAAALR.K
Tender	Myosin-1	R.LEEAGGATSAQIEMNK.K

Tough	Myosin-2	K.NLTEEMAGLDETIAK.L
Tough	Myosin-7	R.IEELEEELEAER.T
T&T	Tropomyosin alpha-1 chain	K.AISEELDHALNDMTSI.-
Tender	Tropomyosin alpha-1 chain	K.CAELEEELKTVTNNLK.S
Tender	Tropomyosin alpha-1 chain	K.HIAEDADRKYEEVAR.K
Tender	Tropomyosin alpha-1 chain	K.KATDAEADVASLNRR.I
T&T	Tropomyosin alpha-1 chain	K.SIDDLEDELYAQK.L
Tender	Tropomyosin alpha-1 chain	R.AQKDEEKMEIQEIQLK.E
Tough	Tropomyosin alpha-1 chain	R.KLVIIESDLERAEER.A
Tender	Tropomyosin alpha-1 chain	R.KYEEVAR.K
Tender	Tropomyosin alpha-1 chain	R.LATALQKLEEAEKAADESER.G
Tender	Tropomyosin alpha-1 chain	R.RIQLVEEELDRAQER.L
T&T	Tropomyosin alpha-1 chain	R.SKQLEDELVSLQK.K
Tender	Tropomyosin beta chain	K.ATDAEADVASLNRR.I
Tender	Tropomyosin beta chain	R.AMKDEEKMELQEMQLK.E
Tough	Troponin C2	R.SYLSEEMIAEFK.A
Tender	Troponin T fast skeletal muscle type	K.DLMELQALIDSHFEAR.K
Tough	Troponin T fast skeletal muscle type	K.ELWDTLYQLETDKFEYGEK.L
T&T	Troponin T fast skeletal muscle type	K.IPEGEKVDFDDIQK.K
Tender	Troponin T fast skeletal muscle type	K.KEEEELVALKER.I

***b) cytoskeletal/organelle proteins*.**

Phosphorylation is most likely on serine (S) or threonine (T) amino acid residues, but it is also possible on tyrosine residues (Y). Peptides which are phosphorylated have a high affinity to TiO_2, but other peptides can be enriched by this method as well as negative charges on a peptide can lead to binding to the tips. This can be observed in the detection of some peptides that contain no serine, threonine or tyrosine amino acid residues, and therefore cannot be phosphorylated

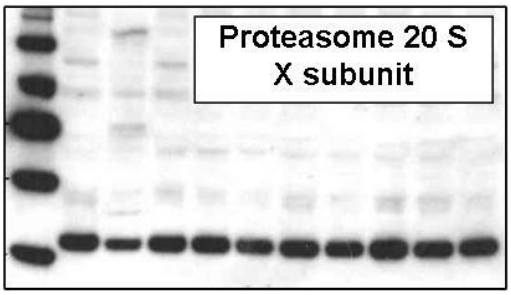
4.4 Validation of Potential Protein Biomarkers by Western Blot Analysis

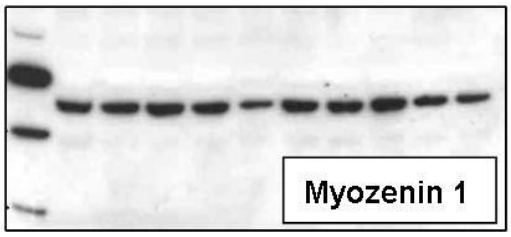
To validate the list of potential biomarkers for initial toughness/tenderness of lamb as reported in tables 2-4, we attempted to measure the amount of the biomarker in the cohort of all 20 samples by Western blot analysis. These values were then correlated against the initial shear force data. Antibodies were sourced against the proteins of interest however there are very few antibodies raised specifically to recognise ovine proteins, therefore where possible antibodies were sourced with cross reactivity across different mammalian species or that specifically reacted to the bovine antigen. We were able to obtain antibodies against 15 different proteins from the list of structural proteins and proteases as shown in the table below.

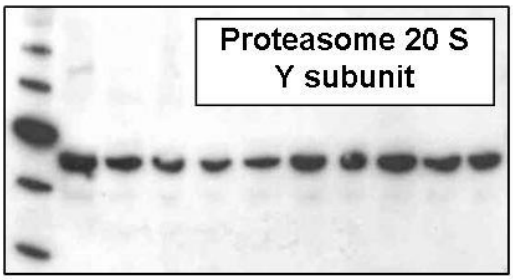
Protein	Species reactivity
Myozenin 1/ Calsarcin	mouse, rat, human, cow

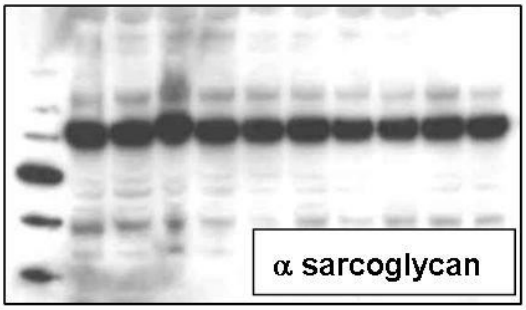
Annexin II	mouse, rat, human, cow
Calsequestrin	Mouse, Ovine, Canine, Rat, Human
Troponin-T	rabbit
Proteasome 20 S Y subunit	human, mouse, cow
Proteasome 20 S X subunit	human, cow
Tropomyosin-4	mouse, rat, human
α sarcoglycan	human
Myozenin 2*	mouse, rat, human, cow
α tubulin*	Mammalian
Myosin*	mouse, rat, human, cow
Annexin IV*	mouse, rat, human, cow
β sarcoglycan*	mouse, rat, human, cow
Calpain	cow
Troponin T-SS*	mouse, rat, human, cow

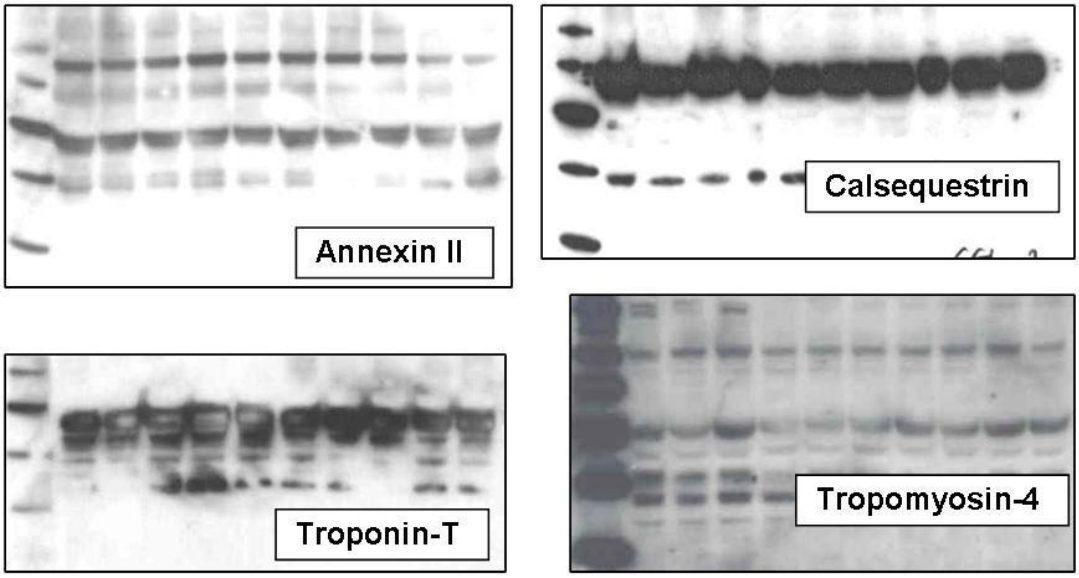
All antibodies were tested against crude homogenates of the muscle samples, if immunoreactivity was detected, the optimal conditions (primary and secondary antibody dilutions) for imuno-reactivity were determined. We were able to successfully optimise the Western blot analysis for 8 antibodies shown in bold; however we were not able to establish the condition for other 7. The major problem was that a number of the antibodies (indicated by an *) were raised in goats and the immunglobulins cross-reacted non specifically to the lamb samples. The 8 optimised Westerns blots were used to measure the level of the protein biomarkers within the homogenates of all the lamb samples (appendix figure a-e). We then verified the bio-marker levels against the initial shear force data to determine that by regression











5 Impact of this Research

The objective of this research was to compare the protein expression of lamb loin samples taken at slaughter from similarly raised lambs at the extreme ends of the spectrum for tenderness from a sample of commercially produced and slaughtered lambs. The aim was to identify the significantly differing elements of the proteome between the tough and tender lamb samples at rigor.

Ultimately, the list of potential markers for initial toughness/tenderness will be verified and confirmed. Differential abundance of the markers across all samples ranging from the toughest to the most tender will be conformed by Western blot analysis using specific antibodies. We aim to correlate protein abundance with toughness to derive a set of proteins that explain variation in initial toughness, or predict a threshold for unacceptable toughness within post-rigour lamb samples. Our ability to correlate our candidate proteins of interest within the range of initial toughness values from our 20 individuals will depend on our ability to source specific antibodies of interest or develop assays for determination of protein abundance or activities. We are beginning to scope the range of available technologies and methodologies to complete this task now in order to streamline this very complex component of the research.

The public release of the fully annotated bovine genome sequence, which is due shortly (late 2005) will provide more information from which to search against and so potentially expand the list of identified markers within the tough and tender groups of *M. Longissimus dorsi* at rigour mortis.

6 Conclusions

Given the natural differences in subjective tenderness of meat derived from animals of similar environmental and genetic background, there is potentially large variation in the proteome of similar animals that contribute significantly to the tenderness of their meat. The tenderness of lamb differs most noticeably in the early stages of age conditioning (up to two days post-mortem) when large variability is evident in similar loin samples (Thompson *et al.*, 2004). This variability diminishes as proteolytic activity continues to degrade proteins in the myofibrillar structure as the meat ages. Common retailing practices deliver lamb to the consumer within two days of slaughter. This may have detrimental effects on the perceived sensory properties of the lamb if cooked and eaten prior to optimal age conditioning of the purchased cuts, particularly if the meat was from an animal of poor tenderness values at slaughter. Objective measurement of the tenderness of loin samples of lambs can be obtained nearing the conclusion of rigor through shear force testing of similarly cooked and treated loin samples. In this way, relatively tough and tender samples of lamb loins prior to the influence of any ageing or protein degradation can be obtained from animals of similar genetic and production backgrounds.

This “tough v tender” model for lamb samples collected at *rigor mortis* could be useful to provide evidence of important protein changes that relate to tenderness and also in identifying protein substrates and determining patterns of protein degradation that relate to shear force and meat tenderness.

The approach of this study was to sample lambs from similar genetic and environmental backgrounds and to determine those animals with divergent results for initial objective tenderness of their loin cuts.

This experiment was commenced in conjunction with a study of protein substrate degradation and proteolytic activity in meat being conducted by the MIRINZ research group of AgResearch in New Zealand. This group had developed a model for studying protein degradation and ageing in meat samples with the aim of understanding proteolysis and improving the sensory properties of meat for consumers.

7 Recommendations

- Optimisation of western blotting detection systems using Qdot® nanocrystal labelled secondary antibodies will provide greater dynamic range and quantitation of differences in protein abundance between samples. It is recommended that this technology be further developed through this project to facilitate new methods of quantitative detection of successful biomarkers through on-line or off-line measurement tools (eg, rapid fluorescent Eliza)

- From the initial list of 55 proteins identified from 2DE and mass spectrometry that had altered relative abundance consistent with differences in eating quality, approximately 20 were characterised as potential targets for development as biomarkers for toughness and tenderness.

Of this list of 20 targets, we have been able to source commercial antibodies to 10. Six antibodies have been optimised for detection of target proteins in sheep muscle, however, 4 require further optimisation or require sourcing alternate antibodies that show appropriate reactivity. It is recommended that additional antibodies to these 4 proteins be sourced and trialled. It is also recommended that further searching be conducted to source antibodies to the remaining 10 protein candidates in the initial list of 20 that we were unable to source antibodies to in our initial scans.

- With sample processing and western blotting conditions optimised for detection of protein abundance using the Qdot® system optimised, we will quickly be able to generate quantitative estimates of protein abundance for the target proteins across the experimental cohort. Final analysis of the individual relationships between proteins and toughness/tenderness will be derived from the system described above. We will provide an estimate of the correlation between protein levels across all targets against initial toughness/tenderness. We will be able to predict within the experimental cohort, what proportion of variation in initial toughness/tenderness is explained by these biomarkers. This result will be outlined in the final report and recommendations for validation and commercialisation will be made.

8 Bibliography

Lo, S. H., Janmey, P. A., Hartwig, J. H. & Chen, L. B. (1994) Interaction of tensin with actin and identification of its three distinct actin-binding domains., *J.Cell Biol. 125*, 1067-1075.

9 Appendices – Tables a-e

Table (a). List of extracellular matrix/myofibrillar proteins with altered relative abundance between tough and tender lamb M. longissimus dorsi at rigor. *Spot numbers refer to proteins identified in Figure 2.*

Spot number	Normalised volume		SEM*	P value
	Tender	Tough		
357	0.821	0.509	.120	.049
401	0.894	1.169	.112	.043
551	1.283	0.754	.173	.042
137	0.644	0.947	.096	.031
578	0.038	0.059	.006	.030
594	0.292	0.396	.031	.023
364	0.065	0.111	.012	.022
521	1.243	1.528	.094	.016
266	0.121	0.277	.049	.011
456	0.027	0.054	.008	.008
342	0.054	0.104	.010	.003
537	0.029	0.062	.005	<.001
462	0.191	-	.031	-
390	0.114	-	.010	-
584	0.699	-	.057	-
179	0.085	0.053	.011	.047
81	0.591	0.722	.045	.041
97	0.487	0.782	.088	.039
116	0.030	0.074	.016	.034
193	0.086	0.056	.011	.033
25	0.813	0.566	.079	.021
98	0.167	0.253	.028	.029
144	0.025	0.038	.003	.010
20	0.417	0.221	.059	.010
241	0.420	0.711	.085	.009
186	0.123	0.078	.013	.007
211	-	0.024	.004	-
264	-	0.039	.008	-
158	0.018	0.025	.003	.049
71	0.717	0.396	.159	.049

***** Standard error of the mean

Table (b). List of heavy structural fraction proteins with altered relative abundance between tough and tender lamb M. longissimus dorsi at rigor*. Spot numbers refer to proteins identified in Figure 3.*

Spot number	Normalised volume		SEM*	P value
	Tender	Tough		
9	0.643	1.030	.140	.046
165	0.406	0.270	.059	.042
237	0.206	0.293	.032	.041
213	0.406	0.270	.059	.041
284	0.276	0.113	.085	.039
235	2.415	1.395	.321	.028
291	0.405	0.551	.044	.023
285	0.096	0.159	.017	.023
286	0.522	0.676	.030	.018
346	0.039	0.067	.008	.010
82	0.123	0.189	.020	.014
142	1.273	2.286	.226	.007
162	1.090	1.566	.131	.007
267	0.531	1.075	.090	.001
79	-	0.020	.002	-
337	0.080	-	.016	-

***** Standard error of the mean

Table (c). List of cytoskeletal/organelle fraction proteins with altered relative abundance between tough and tender lamb M. longissimus dorsi at rigor*. Spot numbers refer to proteins identified in Figure 4.*

Spot Number	Normalised volume		SEM*	P value
	Tender	Tough		
1036	0.088	0.073	.006	.049
663	0.222	0.200	.008	.049
308	0.020	0.005	.007	.049
462	0.092	0.050	.013	.049
328	0.105	0.053	.023	.046
741	0.358	0.262	.032	.046
487	0.342	0.236	.038	.046
766	2.204	1.764	.145	.046
1041	0.038	0.061	.008	.042
563	0.174	0.105	.027	.040
91	0.019	0.010	.003	.039
388	0.041	0.022	.007	.034
928	0.043	0.026	.006	.031
1067	0.051	0.032	.006	.029
320	0.053	0.024	.011	.029
722	0.027	0.010	.008	.028
1064	0.067	0.050	.007	.027
668	1.385	0.964	.150	.026
866	0.035	0.026	.003	.025
1024	0.237	0.507	.083	.024
859	0.152	0.099	.019	.024
339	0.020	0.009	.004	.023
40	0.044	0.021	.008	.023
966	0.069	0.061	.010	.023
854	0.065	0.031	.013	.019
662	4.031	4.824	.263	.017
978	0.687	0.827	.052	.016
708	0.067	0.042	.007	.015
737	0.125	0.067	.015	.012
404	0.132	0.064	.016	.010
334	0.283	0.424	.036	.009
757	0.251	0.045	.065	.007
810	1.370	2.278	.222	.005
803	0.300	0.202	.026	.004

***** Standard error of the mean

Table (d). List of phosphorylated proteins from Fraction 1 (extracellular matrix/myfibrillar) of lambs with high (tough) or low (tender) initial shear force. *Identified by LC MS.*

Num	Protein ID	Tender		Tough	
		Pep Score	Prot Score	Pep Score	Prot Score
1	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta 1.	1.53E-01	312.20	2.33E-01	340.18
2	Bovine 70 Kilodalton Heat Shock Protein	1.10E-03	52.18		
3	binding regulatory protein Gi alpha-2 chain (fragments)	1.64E-03	32.19		
4	3',5'-cyclic-GMP phosphodiesterase (EC 3.1.4.35) 5A	3.04E-01	208.17	4.78E-01	232.17
5	endothelin converting enzyme (EC 3.4.24.-) 1	8.25E-01	208.14	4.85E-01	204.14
6	hexokinase	9.75E-01	280.16	2.96E-01	264.13
7	microtubule-associated protein MAP2 (fragment)	1.00E+00	208.16	1.00E+00	230.13
8	monocyte chemotactic protein bo-MCP-1b	2.51E-03	38.11		
9	interferon-induced RNA-activated protein kinase Inhibitor	5.27E-01	202.15	4.50E-01	200.14
10	alpha 1-antichymotrypsin; ACT			9.63E-03	46.13
11	ATPase, aminophospholipid transporter (APLT)	9.65E-01	258.15	3.48E-02	292.13
12	B Chain- Bovine Bile-Salt Activated Lipase	1.85E-03	78.14	6.70E-03	66.13
13	phospholipase C beta-I form B	7.75E-01	442.18	4.22E-02	416.19
14	cartilage-derived morphogenetic protein 2	6.46E-03	128.13		
15	Bovine Annexin Vi (Calcium-Bound)	5.89E-01	318.21	5.53E-01	310.19
16	Bovine Cytochrome B(5)	2.36E-03	36.14	6.88E-03	32.14
17	bovine protein kinase B	8.02E-02	268.20	2.21E-01	220.21
18	bovine submaxillary mucin 1	9.40E-01	1524.17	9.71E-01	1240.20
19	desmoplakin, desmosomal (fragment)	1.00E+00	324.16	1.00E+00	266.16
20	CD3 protein	7.57E-04	24.12	4.67E-05	38.11
21	cellular retinol-binding protein, CRBP	1.40E-03	24.06		
22	CG7578-PA, isoform A	9.12E-01	266.16		
23	cryptosporidium oocyst wall protein 3, CpCOWP3	1.00E+00	212.16	1.00E+00	218.14
24	cyclic nucleotide gated channel alpha 3	7.80E-01	236.15	6.11E-01	240.14
25	D Chain D, The Structure Of Bovine If1	4.13E-06	30.13	7.82E-03	40.13
26	G Chain G, Beryllium Fluoride Inhibited Bovine F1-Atpase	9.53E-01	350.17	6.94E-01	246.18
27	GABA-A bovine alpha4 subunit	1.00E+00	258.18	4.01E-01	280.16
28	guanine nucleotide-exchange protein	9.39E-01	432.17	3.28E-01	462.18
29	histone H2B	7.46E-01	218.19	1.00E+00	214.11
30	hypothetical lipoprotein	8.92E-01	402.16	1.00E+00	320.14
31	hypothetical polar organelle development protein	5.39E-01	270.18	9.32E-01	282.21
32	JC5734 apolipoprotein A-II - bovine	5.37E-03	10.13	5.01E-03	8.06
33	keratin, 68K type II cytoskeletal, component-fragment	7.94E-03	58.19	1.27E-04	82.17
34	latrophilin 2 splice variant baaae	9.32E-01	304.17	9.73E-01	332.17
35	latrophilin 3 splice variant abaf	1.00E+00	362.18	1.00E+00	310.17
36	lipoprotein B precursor	8.14E-01	320.17	1.36E-01	344.15
37	lipoprotein P67	1.02E-01	278.18	5.73E-01	246.17
38	lysosomal trafficking regulator	9.38E-01	686.24	7.92E-01	604.20
39	maturase	5.91E-03	126.14	2.32E-03	60.13
40	membrane-associated diazepam binding inhibitor;	9.76E-01	234.17		
41	microsomal triglyceride transfer protein	4.51E-01	298.22	8.46E-01	226.16
42	mitochondrial translational initiation factor 2	1.09E-01	350.21	2.33E-01	368.17
43	phosphoinositide-3-kinase, catalytic, alpha polypeptide	1.00E+00	226.16	1.00E+00	226.15

44	potassium channel			9.16E-01	224.20
45	PQ0614 ferritin 2	1.06E-03	14.13	5.83E-04	8.10
46	similar to 1-phosphatidylinositol-4,5-bisphosphate phosphod	9.87E-01	432.19	2.58E-01	394.20
47	similar to 3,5-cyclic-GMP phosphodiesterase	1.00E+00	312.15	1.57E-01	254.15
48	similar to 3-methyl-2-oxobutanoate dehydrogenase	1.00E+00	320.14	9.67E-01	312.15
49	similar to 3-phosphatidylinositol kinase 85K c	6.45E-02	474.15	9.98E-01	522.14
50	similar to 6.8K proteolipid protein, mitochondrial	9.05E-01	218.12	8.98E-01	252.14
51	similar to adenylate cyclase brain	1.00E+00	710.15	8.10E-01	702.18
52	similar to basic fibroblast growth factor precursor	2.02E-01	272.16	8.50E-01	224.12
53	similar to beta-adrenergic-receptor kinase	1.00E+00	206.17	8.63E-04	34.10
54	similar to bovine p57			1.00E+00	230.14
55	similar to collagen alpha 1(XI) chain			6.54E-01	236.16
56	similar to collagen alpha 3(IV) chain	8.44E-01	284.16	7.48E-01	404.14
57	similar to GTP-binding protein GL1 alpha chain -	8.68E-01	234.18	9.24E-01	220.18
58	similar to GTP-binding protein smg-25C	1.00E+00	246.13	1.00E+00	270.15
59	similar to H+-transporting two-sector ATPase (	9.87E-01	322.20	8.53E-01	324.17
60	similar to interphotoreceptor retinoid-binding protein prec	1.00E+00	244.16	1.00E+00	216.16
61	similar to NADH2 dehydrogenase (ubiquinone)	1.76E-01	228.14	7.96E-01	230.14
62	similar to nicotinic acetylcholine receptor delta chain pre			1.00E+00	258.13
63	similar to nonhistone chromosomal protein HMG-14 -	3.78E-01	264.14	1.00E+00	238.15
64	similar to peptidylglycine monooxygenase	6.60E-01	228.13	9.86E-01	274.16
65	similar to protein kinase C alpha	1.54E-01	206.17	3.89E-01	280.14
66	similar to synaptotagmin I	1.98E-03	94.14	7.97E-03	126.14
67	putative response regulator component	1.00E+00	212.15	1.00E+00	208.15
68	RAS p21 protein activator	7.51E-01	328.20	1.55E-01	340.17
69	S32369 gamma-SNAP protein	7.11E-01	214.12		
70	S32383 cathepsin D precursor	8.45E-05	40.19	6.88E-04	26.19
71	S39346 unc-18 protein homolog, 67K	9.78E-01	392.18	8.73E-01	436.20
72	S41749 myosin heavy chain I beta	9.37E-01	324.17	1.97E-01	278.14
73	S65741 1-phosphatidylinositol 4-kinase type 3	8.79E-01	302.17	8.22E-01	276.17
74	similar to D-aspartate oxidase	1.94E-01	268.20	8.85E-01	312.17
75	supervillin; P205	2.37E-01	770.16	5.35E-01	816.19
76	T11578 probable lipoxygenase	2.82E-01	224.19		
77	Tenascin-X	8.67E-01	496.18	1.00E+00	512.17

Table (e). List of phosphorylated proteins from Fraction 3 (cytoskeletal/organelle) of lambs with high or low initial shear force. *Identified by LC MS*

Num	Protein ID	Tender		Tough	
		Pep Score	Prot Score	Pep Score	Prot Score
1	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta	9.51E-01	366.18	1.00E+00	298.17
2	hexokinase	3.86E-01	228.12	9.52E-01	234.15
3	aspartate beta-hydroxylase	8.59E-06	118.19	6.29E-04	164.17
4	phospholipase C beta-I form B	8.94E-01	352.15	2.78E-01	334.17
5	Bovine Annexin Vi (Calcium-Bound)	5.57E-01	230.18	3.74E-01	296.16
6	bovine C4BP alpha chain	3.12E-03	12.10	2.87E-03	66.12
7	bovine leukocyte antigen	9.90E-03	26.11	9.00E-03	32.09
8	bovine submaxillary mucin 1	1.00E+00	1004.16	1.00E+00	1616.18
9	desmoplakin, desmosomal (fragment)	1.00E+00	226.13	8.63E-01	342.16
10	CD3 protein	1.15E-03	66.15	1.87E-03	46.13
11	CG7578-PA, isoform A	8.10E-01	282.14	8.22E-01	290.15
12	cleavage and polyadenylation specific factor 1, 160kDa	1.00E+00	228.15	1.00E+00	272.14
13	COX5B_BOVIN Cytochrome c oxidase polypeptide Vb (VI)	3.68E-05	46.14	7.20E-03	52.16
14	cryptosporidium oocyst wall protein 2	1.35E-01	218.12	1.00E+00	232.14
15	D Chain D, The Structure Of Bovine If1	9.38E-06	38.18	3.11E-05	68.18
16	Wild-Type Anionic Trypsin Complexed With Pancreatic Tryps	9.84E-04	24.15	7.45E-06	22.16
17	GABA-A bovine alpha4 subunit	1.00E+00	204.14	9.85E-01	344.18
18	guanine nucleotide-exchange protein	6.01E-01	318.16	7.46E-01	352.18
19	H Chain Structure Of Mitochondrial Cytochrome Bc1 Complex	1.94E-05	12.14	3.12E-06	20.12
20	heat shock 10kD protein			9.27E-03	26.15
21	hypothetical lipoprotein	1.00E+00	272.18	7.57E-01	298.17
22	hypothetical polar organelle development protein	8.87E-04	154.13		
23	KABOSB alpha-s1-casein precursor	2.66E-09	34.25	8.76E-11	22.26
24	latrophilin 2 splice variant baaae [	1.00E+00	284.16	9.99E-01	402.18
25	latrophilin 3 splice variant abaf [	9.76E-01	318.13	7.10E-01	386.16
26	lipoprotein B precursor	5.66E-01	256.17	1.00E+00	278.13
27	lysosomal trafficking regulator	1.00E+00	510.16	5.44E-01	608.18
28	microsomal triglyceride transfer protein	7.90E-01	244.15	1.00E+00	264.15
29	mitochondrial translational initiation factor 2	1.18E-01	220.16	5.63E-01	246.14
30	p64 bovine chloride channel-like protein			9.95E-03	38.15
31	phase 1 flagellin	8.33E-01	236.15	1.00E+00	276.14
32	similar to 1-phosphatidylinositol-4,5-bisphosphate phosphod	8.17E-02	360.19	1.00E+00	322.15
33	similar to 3-methyl-2-oxobutanoate dehydrogenase	1.00E+00	364.15	1.00E+00	228.13
34	similar to 3-phosphatidylinositol kinase	9.65E-01	498.16	8.72E-01	444.16
35	similar to adenylate cyclase	9.95E-01	688.17	8.20E-01	748.18
36	similar to basic fibroblast growth factor precursor	9.70E-01	204.15	1.00E+00	204.12
37	similar to collagen alpha 3(IV) chain - fragment	1.00E+00	256.16	2.04E-01	258.16
38	similar to H+-transporting two-sector ATPase	1.00E+00	240.17	8.99E-01	240.16
39	similar to interphotoreceptor retinoid-binding protein prec	8.25E-01	224.17	9.61E-01	242.16
40	similar to nonhistone chromosomal protein HMG-14	1.00E+00	338.15	9.90E-01	276.18
41	similar to peptidylglycine monooxygenase	6.64E-01	228.15	1.00E+00	278.12
42	similar to protein kinase C alpha	2.93E-01	286.16	1.09E-01	238.14
43	putative response regulator component	1.00E+00	218.13	1.00E+00	226.15
44	R Chain Heart Cytochrome C Oxidase - Fully Reduced State	5.82E-08	28.20	1.19E-06	20.17
45	NADH2 dehydrogenase (ubiquinone) (	5.91E-08	18.28	8.67E-03	36.11
46	NADH2 dehydrogenase (ubiquinone)chain CI-15	1.54E-03			
47	S39346 unc-18 protein homolog, 67K			5.62E-01	258.17

48	S41749 myosin heavy chain I beta - bovine	7.68E-01	220.16		
49	1-phosphatidylinositol 4-kinase (EC 2.7.1.67) type 3-fragment	1.00E+00	240.14	6.47E-01	276.14
50	supervillin; P205 [Bos taurus]	8.96E-01	264.15	1.96E-03	568.18
51	Tenascin-X [Bos taurus]	9.96E-01	642.16	3.58E-02	410.17