final report

Project Code: P.PSH. 0225

Prepared by: Clyde Day & Nicola Simmons
AgResearch

Date published: December 2006

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Predicting tenderness from proteolytic enzyme activity in the live animal and on-line carcase

This is an MLA Donor Company funded project.

Meat & Livestock Australia and the MLA Donor Company acknowledge the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

The process of meat tenderisation is highly variable even when production and processing conditions are tightly controlled. Although there are substantial commercial advantages to be gained by producing a consistent level of tenderisation, the only reliable method of reaching this goal at this time is to increase the duration of the aging period, which ensures that the ultimate level of tenderness is attained before the product is sold, since ageing rate is a greater source of variability than ultimate tenderness. However, long ageing periods increase cost and are not normally a viable option for the majority of markets.

The source of variability is generally attributed to differences in the rate of proteolytic degradation of structural myofibrillar proteins within the meat. It is also generally believed that the activity of proteolytic enzymes, in particular the calpain proteases, is responsible for the differences in the rate of proteolytic degradation. A further possibility to explain variable rates of proteolysis is the state of the substrate proteins. The possibility that substrate susceptibility is a source of variability in the rate and extent of meat tenderisation is being explored.

The main challenge in addressing the question of variable susceptibility is identifying and developing a sensitive and rapid assay system. The approach being investigated in this research is to a competitive assay. A protease substrate that fluoresces when degraded is introduced into an assay system together with the protease and endogenous substrate (in this case, myofibrils): under equivalent conditions of substrate and protease concentration, a higher rate of fluorescence development reflects a less competitive endogenous substrate and, hence, a lower affinity between the endogenous substrate and the protease.

The current research found that the calpain fluorescence assay demonstrated effective tenderness prediction, and also interactions with the levels of heat shock proteins. Of particular interest were the results of the protein substrate susceptibility assay, which shows that the rate of tenderisation depends on the state of the structural proteins that are degraded by the calpain enzyme, and is not solely dependent on the enzyme activity itself.

A procedure to carry out ELISA's for both HSP 20 and 27 has been developed. This procedure is currently being refined and validated to establish the Standard Operating Procedure (SOP). The SOP will then be used to measure assay reproducibility, sensitivity and linear dynamic range. Once complete, the ELISA's will be used for routine screening of samples.

The use of HSP to assess pre-slaughter stress and optimise pre-slaughter handling system is currently underway. Further research is required to evaluate a commercial tool in the following areas:

- develop muscle biopsy procedures to allow routine on-farm measurements of calpain and HSP activity
- Identify production and pre-slaughter management contributions to 24 hour tenderness
- Identify sources of variable substrate susceptibility to calpain degradation and tenderisation

Executive Summary

The process of meat tenderisation is highly variable even when production and processing conditions are tightly controlled. Although there are substantial commercial advantages to be gained by producing a consistent level of tenderisation, the only reliable method of reaching this goal at this time is to increase the duration of the aging period, which ensures that the ultimate level of tenderness is attained before the product is sold, since ageing rate is a greater source of variability than ultimate tenderness. However, long ageing periods increase cost and are not normally a viable option for the majority of markets.

The source of variability is generally attributed to differences in the rate of proteolytic degradation of structural myofibrillar proteins within the meat. It is also generally believed that the activity of proteolytic enzymes, in particular the calpain proteases, is responsible for the differences in the rate of proteolytic degradation. However, the evidence for this is not particularly strong: purification and quantification of the calpains or the specific calpain inhibitor, calpastatin, have explained a proportion of the variability found in the normal range of commercial livestock (although better explanations are found when considering experimentally-induced toughening (β-agonist administration) or genetic (callipyge gene) models).

A further possibility to explain variable rates of proteolysis is the state of the substrate proteins. Post-translational modification of proteins is known to modify their interactions with a range of enzyme systems, including proteases. Indeed, such translations, in particular protein phosphorylation, provide a much more sensitive and targeted manipulation of substrate flux through enzyme pathways than modification of the enzymes themselves. The possibility that substrate susceptibility is a source of variability in the rate and extent of meat tenderisation is being explored.

The main challenge in addressing the question of variable susceptibility is identifying and developing a sensitive and rapid assay system. Our approach has been to develop a competitive assay. A protease substrate that fluoresces when degraded is introduced into an assay system together with the protease and endogenous substrate (in this case, myofibrils): under equivalent conditions of substrate and protease concentration, a higher rate of fluorescence development reflects a less competitive endogenous substrate and, hence, a lower affinity between the endogenous substrate and the protease.

To develop this concept, trypsin was used as the protease and myofibrils were used in a washed form in the assay system. A significant difficulty with this assay was the effect of purification and washing of the myofibrils: as the myofibrils are washed, they develop a strong tendency to aggregate, and the extent of aggregation significantly affects the rate of degradation. Only very careful and time consuming preparation procedures were found to produce reproducible results, but this requirement subtracted from the objective of a rapid and simple assay system. Because of these difficulties, the next stage in the development of the susceptibility assay - changing to a calpain protease, did not get beyond a preliminary stage. However, an alternative approach does present itself. Since a crude homogenate contains both the required calpain system and the myofibrils in their native, unwashed state, there is the potential to set up the conditions for a competitive assay within the crude homogenate. These conditions would meet the requirements of a simple and rapid assay.

Table of Contents

1 Background

The rate and extent of proteolysis in meat after slaughter defines the development of tenderness and remains a highly variable attribute of meat. Understanding the sources of variation and manipulating proteolysis to ensure rapid tenderisation would have important commercial implications for the meat industry by, first, reducing the time of chilled storage necessary to ensure acceptable tenderness and, second, avoid the incidence of tough meat that creates to consumer dissatisfaction.

Existing methods to study proteolysis in meat are based on elaborate purification procedures that, ultimately, quantify the amount of enzyme present but offers no indication of how much proteolysis is actually taking place in the meat. This is because enzymes are not always in an activated form, are compartmentalised and/or inhibited by specific endogenous inhibitors. The procedure under development in this objective quantifies the activity a whole tissue homogenate while trying to minimise artifactual activation or inactivation of the enzyme activity, to provide a direct index of the proteolytic activity, rather than the amount of enzyme. Commercially available fluorescent substrates are added to crude homogenates and total activity measured by monitoring changes in fluorescence activity associated with degradation of the substrate. Because the synthetic fluorescent substrates are designed for specificity to specific protease enzymes, the activity of different enzymes in meat samples can be measured independently of each other by appropriate choice of fluorescent substrate.

The objectives of developing this assay are two-fold: first, we propose to adapt the assay for use as an on-line predictor of tenderness. Because of the limited sample preparation and the ease with which a fluorescence-based assay can be used in samples with minimal purification, this assay is ideally suited for an on-line and automated measuring system.

The second objective is the measure the tenderness potential of animals without resorting to slaughter, by the measurement of enzyme activity from biopsies. The purpose of this is to identify genetic and on-farm practices that will affect enzyme activity and, hence, the ability to tenderise. Of particular interest is the opportunity to measure the time course of changes in enzyme activity in response to environmental factor that are believed to affect meat tenderness.

The purpose of the current research is to evaluate additional protease systems for their ability to predict variations in tenderness and assess the commercial opportunities of on-line protease measurements. Specifically, the research will report on contribution of alpha-crystallin concentrations to the natural variations in beef tenderness attributes. Furthermore, an ELISA assay for alpha-crystallin will be developed for use as a marker for pre-slaughter stress and post-mortem tenderisation. It is expected that the outcome of this research will be a commercial measurement for tenderness.

The proposed outcome of this research is a description of the methodology to measure the proteolytic activity in muscle and meat; a critical assessment of its ability to predict proteolysis and, hence, the development of tenderness; potential applications for the technology in an industrial context.

2 Project Objectives

The objectives of the current research were:

- In combination with the protease and susceptibility assays, report on the contribution of low molecular weight HSP concentrations to the natural variations in beef tenderness attributes.
- Complete the development of the myofibrillar susceptibility assay.
- Development of an assay that can be used to predict tenderness.
- Guidelines on the use of the assay as an on-line tool to predict the tenderness of each carcass and as a tool to predict the tenderness potential from a biopsy sample of a live animal.

3 Methodology

3.1 In combination with the protease and susceptibility assays, report on the contribution of low molecular weight HSP concentrations to the natural variations in beef tenderness attributes.

This objective has evaluated the opportunity to measure the activity of calpains and other proteolytic enzymes by measuring the degradation of protease-specific substrates that fluoresce as they degrade. The principle of this assay is to develop a very simple and rapid procedure, with minimal sample preparation beyond homogenisation, and allow the combined interaction of activating and inhibiting factors present in the sample to produce a resultant activity that will represent the summation of proteolytic events responsible for tenderness.

In the evolution of this assay, a number of different substrates have been evaluated, and the calpain activity in ruminants was found to be best represented by two substrates, CalS1 and CalS3. CalS1 is specific to µ-calpain, whereas CalS3 is degraded by µ- and m-calpain. Recently, the assay procedures were modified from previous milestones to allow a whole homogenate to be used, rather than just a supernatant as previously described. This modification avoids possible loss of enzymes and enzyme activity modifiers that are potentially associated with the myofibrillar fraction. It also permits the opportunity to undertake a substrate susceptibility assay, as described in a previous milestone. A substrate for Cathepsin B was also evaluated.

Earlier milestones have reported that the calpain enzymes appear to be bound to low molecular heat shock proteins, HSP 20 and HSP 27. This relationship was identified by immunoprecipitation, and in vitro assays, and showed that the presence of HSPs could influence the kinetics of proteolytic degradation. Moreover, the levels of HSP expression in muscle tissues were influenced by pre-slaughter stress, so this discovery offered a mechanistic explanation for a relationship between pre-slaughter stress and meat quality.
This milestone simultaneously measured the calpain and cathepsin activities, HSP levels and tenderness characteristics in an effort to explain the normal (and substantial) variability found in beef striploins.

Ten striploins were collected during the early pre-rigor period, from a commercial hot boning plant. The muscles were immediately immersed in 15°C water and maintained at this temperature throughout the pre-rigor and aging periods. pH measurements were repeatedly

made to identify rigor mortis (pH<5.7), which was taken as time 0 in the ageing curve. Shear force was measured at days 0,1,2,3 and 5. This was repeated on 4 occasions (the results of the 5th week are being completed) providing a total of 40 samples.

The calpain and cathepsin measurements were made at kill and at rigor mortis, using the same methodology described previously. The assays were carried out at pH 5.5, using a saturating concentration of substrate and a sub-maximal concentration. The substrate susceptibility was calculated as the ratio of the submaximal and maximal substrate concentrations.

HSP 20 and 27 were analysed using E-page Western Blots. The ELISA assays for these substrates has been delayed because of unavailability of a necessary antibody, but the samples will be tested by this method when the antibodies arrive. In the meantime, the western blots will provide a semi-quantitative measure of the HSP concentrations. Each sample was measured in triplicate and expressed as an average having been quantified using densitometry.

3.2 Complete the development of the myofibrillar susceptibility assay

In order to demonstrate the possibilities of this approach, a qualitative assessment of the principles was made using mathematical modelling. The calpain protease is assumed to behave as a simple enzymatic reaction with the calpain-specific fluorescent substrate (CalS3): an initial equilibrium reaction between the enzyme and substrate to form an intermediate complex, which can also, as a mass action reaction, convert irreversibly to a degradation product and free enzyme. An equivalent reaction was defined for the reaction between the protease and myofibrils.

The equilibrium reaction between the calpain and CalS3 was set at 2 orders of magnitude greater than the reaction between the calpain and myofibrils. The molar concentration of myofibrils was set at 1000µM, the enzyme concentration at 1µM and the CalS3 concentration at 20µM.

Meat samples were homogenised in 50mM Tris-MES buffer, pH 5.5, containing 100 mM KCL, 1 mM 2- mercaptoethanol NS 0.1mM PMSF. Typically, the homogenate was adjusted to a final concentration of 1 g/ml. 30µl homogenate was added to CalS3, a calpain substrate that is degraded by both milli-calpain and micro-calpain, to varying concentrations as described in the individual experiments, and a further 50µl assay (homogenisation) buffer. The reaction was carried out in a 96 well plate and the rate was measured using a fluorescence plate reader.

3.3 Development of an assay that can be used to predict tenderness (ie guidelines on the use of the assay as an on-line tool to predict the tenderness of each carcass and as a tool to predict the tenderness potential from a biopsy sample of a live animal).

ELISA assays were performed in 96-well polystyrene plates with flat bottoms (Maxisorp). The bottom of the wells were coated with two different concentrations of anti-HSP antibody (either anti-HSP20 or anti-HSP27) diluted in one of two buffers [phosphate-buffered saline(PBS) or bicarbonate buffer, pH 9.4] by incubating overnight at 4°C. The antibody solutions were then aspirated off and all available protein binding sites on the plates blocked using a casein solution in PBS by incubating for one hour at 37°C.

All subsequent incubations were done at 37°C for one hour with the reagents diluted in blocking solution. After aspirating off the blocking solution, the plate was incubated with a four log concentration range of purified human HSP to determine assay sensitivity and range. The wells were washed with PBS containing Tween20, followed by incubation with four different concentrations of a second anti-HSP antibody. The wells were washed and the plates incubated with two different concentrations of horseradish peroxidase-conjugated secondary antibody.

Binding was measured using three different peroxidase substrates that result in a colored product.

3.4 Report on interaction of low molecular weight HSP with post mortem tenderisation and meat quality

The M. longissimus dorsi muscles were collected pre-rigor from 104 prime carcasses. Typically, 10 striploins were collected on any given day, and immediately cooled in 15°C water through the pre-rigor period. Pre-rigor pH measurements were made at intervals to determine when the samples reached rigor mortis (ultimate pH). Once the samples reached rigor, a sub-sample was cut and cooked prior to shear force measurement – this provided a tenderness measure at rigor. The samples were subsequently maintained at 15°C during the ageing period and shear forces measured at intervals to define both the ageing rates and ultimate tenderness. Ageing rates were calculated by fitting an exponential curve, and expressed as the time constant of the fitted curve
(-hour). In addition to tenderness, water binding capacity (WBC) was measured at rigor using he filter paper press method, and unbloomed and bloomed colour were measured.

In addition to the 15°C pre-rigor treatment, 60 duplicate samples (contralateral striploin) were also collected on 4 separate collection dates and these samples were held at 40°C through the pre-rigor period, then subsequently aged at 15°C. Tenderness parameters (initial and final shear force, and calculated ageing rate), WBC and at-rigor colour parameters were measured as described for the 15°C samples.

The HSP 20 and 27 concentrations in the samples were measured using the E-Page western blot method. The samples were collected at kill, snap frozen in liquid N2 and stored frozen until analysed.

3.5 Using HSP's as a quantitative measure provide a preliminary report outlining some of the best practice methods for stock presentation at point of slaughter

Samples from the M.longisimus dorsi were collected at the end of the dressing operation within 20 minutes of slaughter. At least two separate treatments were identified on each collection and 10 samples were collected from each treatment on each collection. A total of 100 samples were collected.

After excision from the carcass, the samples were snap frozen in liquid nitrogen and held at -25°C for a maximum of three weeks prior to analysis. HSP20 was measured by ELISA according to the methodology outlined in a previous milestone. The methodology for HSP27, although accurate against the purified standard, proved to be inconsistent in the meat homogenate, and while a range of revised procedures are currently being evaluated to improve the performance of this assay, in order to complete this milestone, HSP27 was

quantified using the E-Page western blot method, in triplicate and using a HeLa cell lysate as an HSP27 standard.

4 Results and Discussion

4.1 In combination with the protease and susceptibility assays, report on the contribution of low molecular weight HSP concentrations to the natural variations in beef tenderness attributes.

4.1.1 Tenderness:

The tenderness attributes of the samples during the early post-mortem period showed the usual substantial variability. Initial values varied between 11 and 23 KgF, while the ultimate tenderness after ageing varied between 1.7 and 6.5 KgF. The rate of ageing, as defined as the rate constant of an exponential fit through the data points, ranged from 0.018 and 0.078days^{-1}. These ranges are in keeping with previous experiments.

4.1.2 Calpain activity:

We found a significant batch-related variability with the CalS3 substrate and the results of this substrate could not be used. The CalS1 activity was effectively described by an exponential curve and the initial rate of the reaction was calculated from the fitted curve. The cathepsin activity was linear over the first 30 minutes of the measurement and the slope was therefore calculated from a linear regression.

4.1.3 Correlations:

The cathepsin substrate did not offer any significant explanation for the tenderness attributes. The main correlation with the ageing rate came from the at-kill CalsS1 susceptibility assay. The correlation coefficient (r^2) was 0.52 ($p<0.001$) reflecting that high levels of substrate susceptibility results in increased ageing rate (see Figure 1).

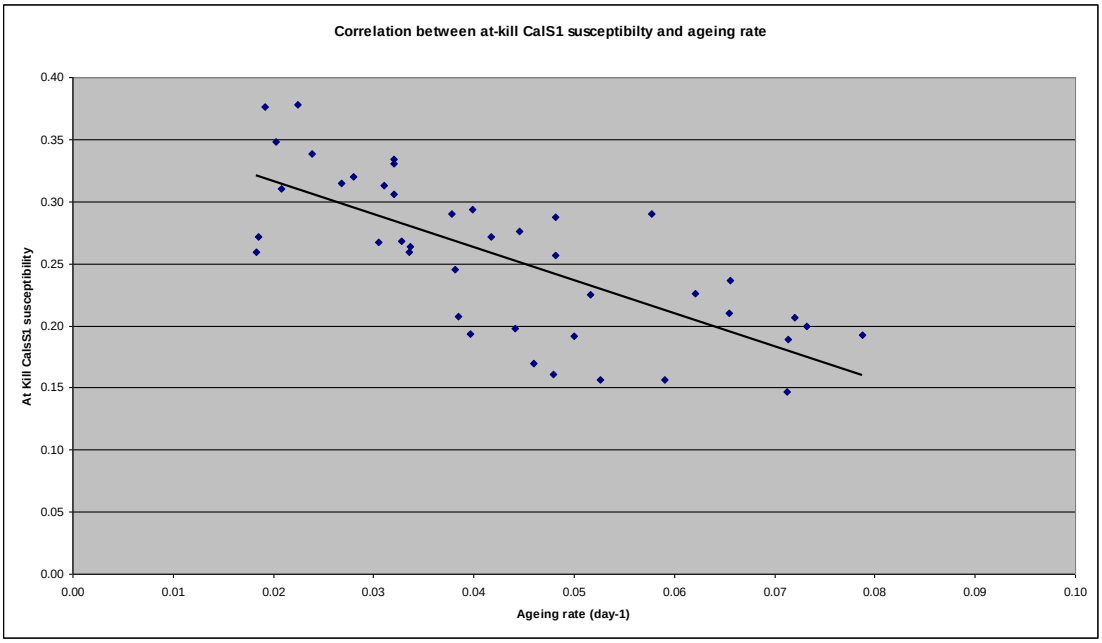

Figure 1: Correlation between at kill CalS1 susceptibility and ageing rate.

The initial tenderness (at rigor) was most highly correlated with the at-rigor CalS1 susceptibility (R2 = 0.29; $p<0.001$). Again, the higher the calculated susceptibility, the lower the at-rigor shear force.

The final tenderness was primarily correlated with the maximum CalS1 activity at rigor (R^2=0.28; p<0.001). The correlation with CalS1 activity at kill was also highly significant (p=0.003).

4.1.4 HSP measurements.

The HSP20 was significantly correlated with HSP27 concentration (r^2=0.45; p<0.001). Since the western blots for the two proteins use different antibodies and were carried out on separate gels, this relationship suggests that the quantification was reasonably meaningful.

HSP 20 proved to have the closest relationship with calpain enzyme activity. HSP activity was highly correlated with maximum CalS1 activity both at kill and at rigor, and with submaximal activity at kill and at rigor (P <0.001 – 0.005). The highest correlation was with at-kill maximum CalS1 activity (r^2=0.48).

HSP activity was also highly correlated with final tenderness (r^2=0.42; p<0.001).

Using multiple regressions combining the measured HSP activity with the measured calpain activity to predict tenderness attributes, did not show a significant improvement over the calpain activities alone. The implication of this finding is that the effect of the HSP on tenderness attributes is due to impact of HSP on mediation of the calpain activity.

The implication of these results is that the modified calpain assay offers some important insights into the determinants of tenderness attributes. Of particular interest is the importance of the susceptibility assay, which would seem to suggest that the state of the protein substrate has an important role in the rate of tenderisation, rather than simply the state of the enzyme system. A confirmation of the HSP measurements using the ELISA assay will be included in the analyses to confirm these conclusions.

4.2 Development of an assay that can be used to predict tenderness.

The effect of increasing the concentration of CalS3 between 1 and 1000µM (in 20 equal increments) is shown in Figure 2. As expected, the initial reaction rate increases with concentration of CalS3 until the CalS3 is present in excess, which results in no further increase in the initial rate. Calculated initial slopes are shown in Figure 3.

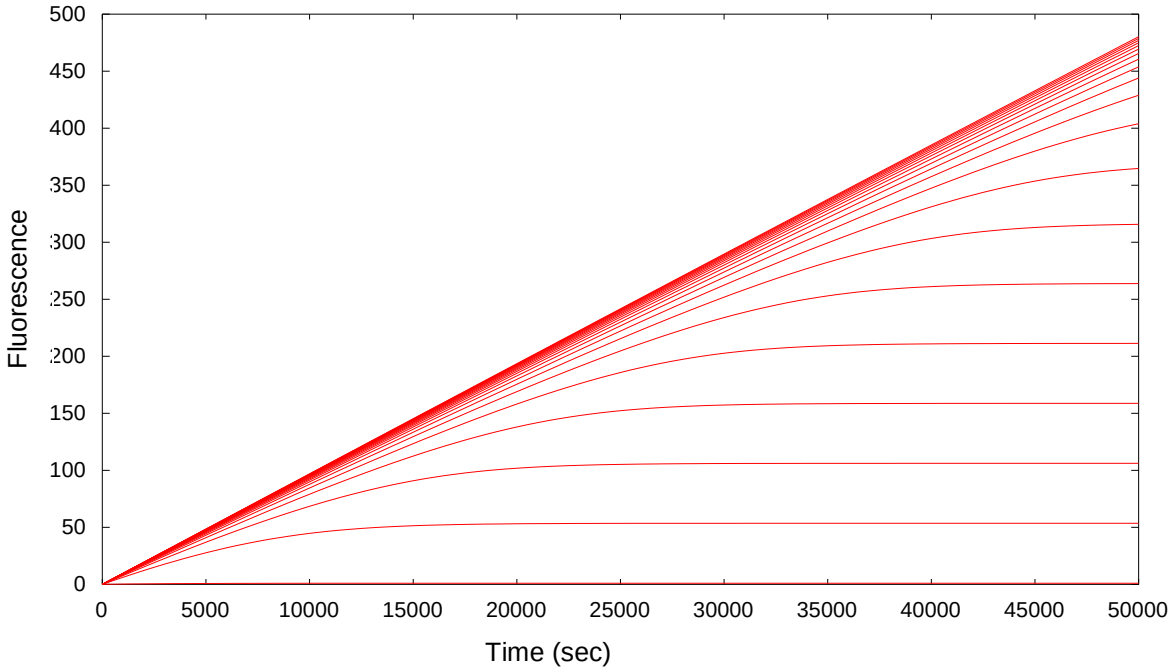

Figure 2: Calculated effect of increasing CalS (1-1000 µM) in CalS3 degradation.

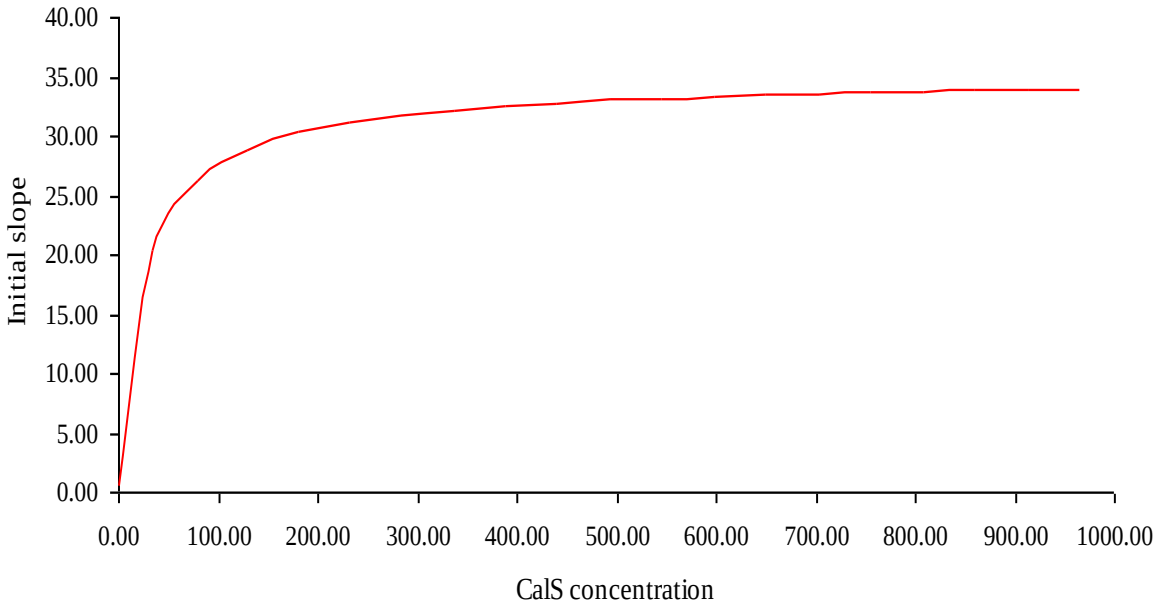

Figure 3: Calculate effect of increasing CalS (1-1000 µM) on initial rate of reaction.

Change in the substrate susceptibility to proteolytic degradation can be expressed as a change in the equilibrium constant controlling the interaction of calpain with the myofibrils.

The effect of changing the equilibrium constant between 1e-4 and 1e-3 (in 20 equal increments) on the rate of degradation of CalS is shown in Figure 4: as the equilibrium constant between the calpain and myofibrils increases (increased affinity), the competition with CalS increases and the rate of fluorescence decreases accordingly.

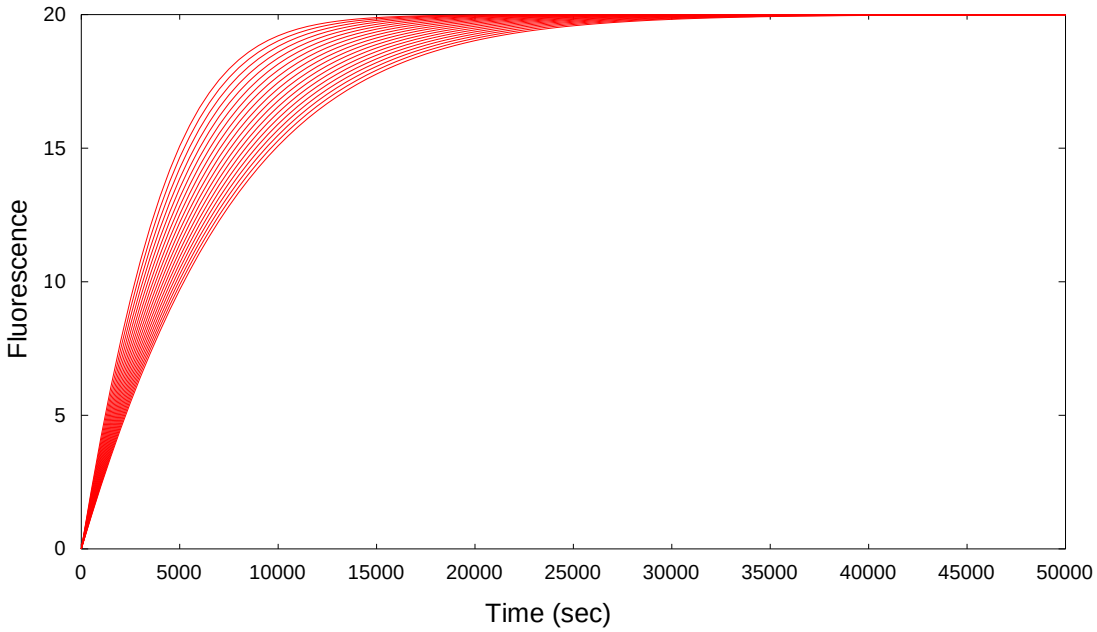

Figure 4: Calculated effect of changing myofibrillar susceptibility on rate of CalS degradation.

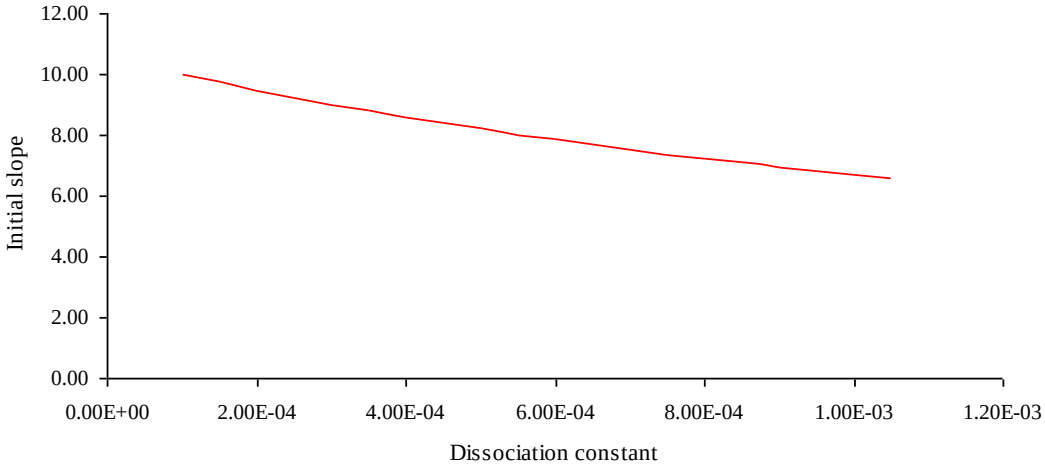

Figure 5: Calculated effect of changing myofibrillar susceptibility on initial rate of CalS degradation.

The effect on the rate of myofibrillar degradation is shown in Figure 6.

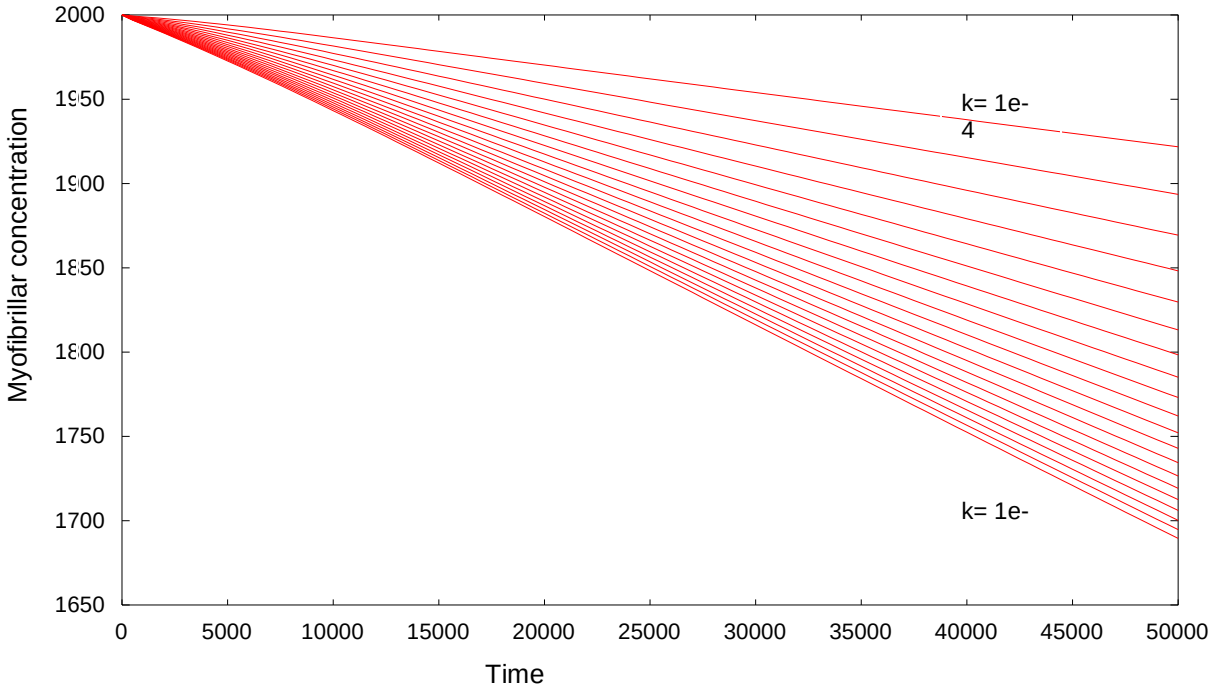

Figure 6: Calculated effect of changing myofibrillar susceptibility on the rate of myofibril degradation.

An example of the effect of varying CalS3 concentrations is shown in Figure 5. The CalS3 concentrations were 1, 2.5, 5, 10 and 20µM. Figure 7 shows the calculated initial slopes, averaged from 4 separate meat samples, collected near rigor following a 15 °C pre-rigor incubation.

The results show a response comparable to those predicted from the modelled reaction. The activity increased in a dose dependent manner until a saturation level was reached (approximately 60µM).

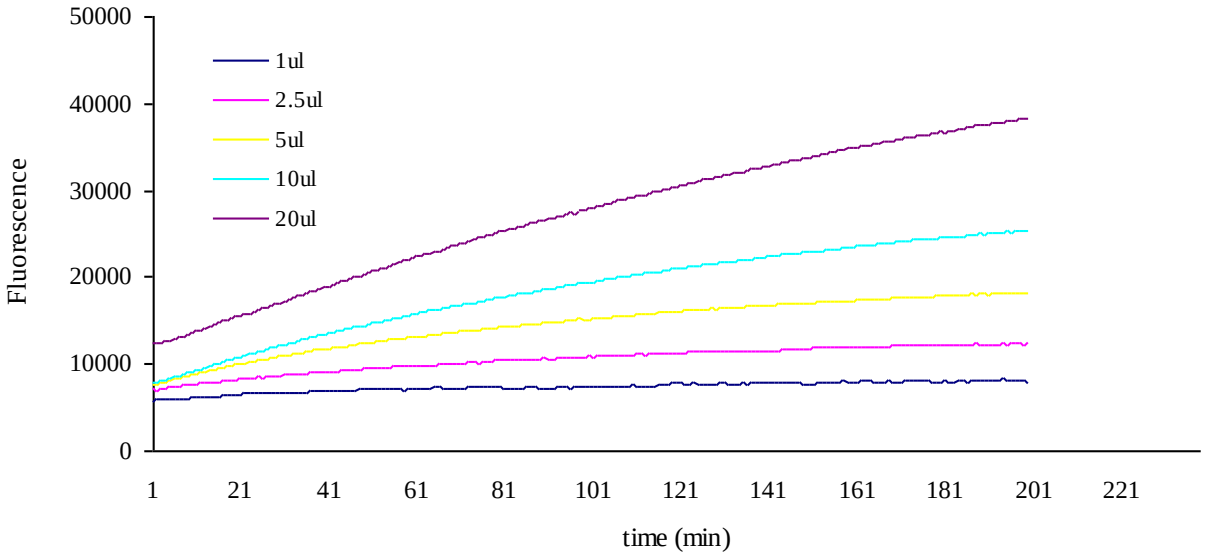

Figure 7: Measured rate of CalS3 degradation at different CalS3 concentrations

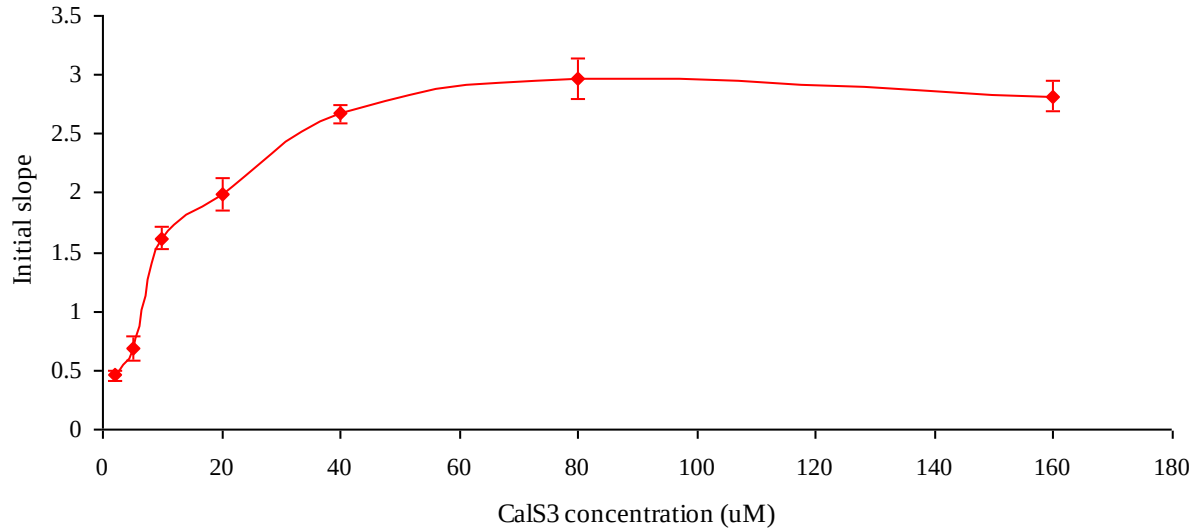

Figure 8: Measured mean (+/- S.D.) initial slope at different CalS3 concentrations (n=4)

Defining the range of variation in myofibrillar susceptibility and the potential benefit in explaining variation in meat tenderisation will be the objective of the next milestone. However, it is recognised that protein denaturation can have a significant effect on the rate of proteolytic degradation, and denaturing processing conditions (high temperatures combined with low pH) not only results in the denaturation of myofibrillar proteins but can also have a significant effect on meat tenderness. Additionally, striploins from 2 animals were incubated during the pre-rigor period at either 15°C or 40°C, then prepared for proteolytic activity and myofibrillar susceptibility within 6 hours of *rigor mortis*.

A comparison of the activity measured at a range of CalS3 concentrations was made in these samples and the results are shown in Figure 9. Sample 2 shows relatively little effect of pre-rigor incubation temperature, but the failure to reach a saturating concentration of CalS3 before 80µM suggests a very high calpain activity in this sample.

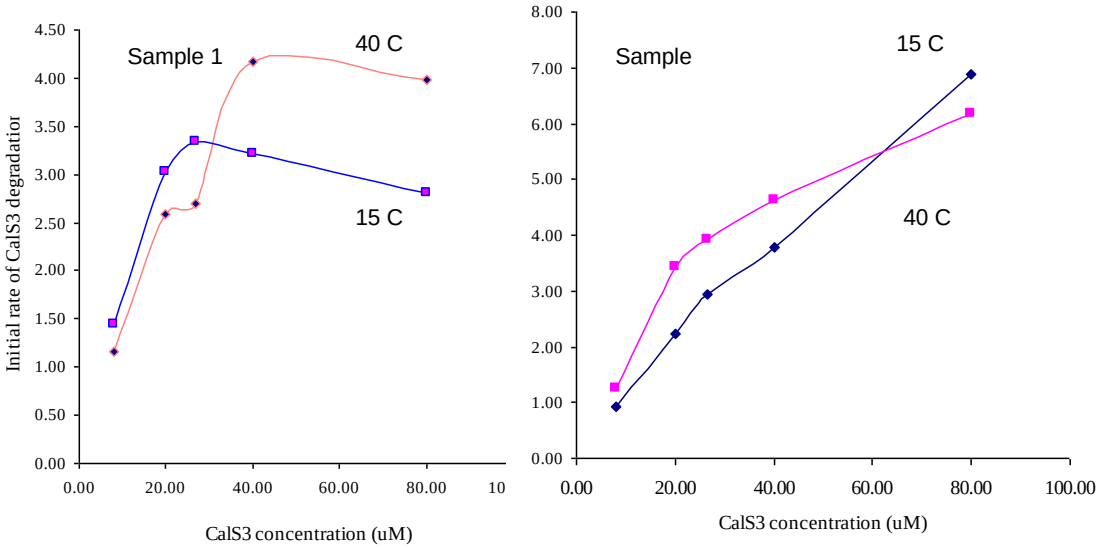

Figure 9: Comparison of CalsS3 degradation

In contrast, Sample 1 reaches saturation at 30µM at 15°C, and 40µM at 40°C. The greater peak calpain activity in the high temperature treatment is somewhat surprising, although our earlier work has shown that calpastatin activity degraded more rapidly at high temperatures. Although the CalS3 concentration-dependence of the degradation rate appears similar in both temperature treatments, this activity needs to be corrected for the greater total calpain activity in the high temperature treatments.

By normalising the activity at each concentration for the saturated activity, the slope describing the initial rate of CalS3 degradation against CalS3 concentration is 0.022 for the high temperature treatment, compared with 0.032 for the low temperature treatment. The implication is that the substrate susceptibility is greater in the denatured, high temperature-treated sample.

4.3 Development of an assay that can be used to predict tenderness (ie guidelines on the use of the assay as an on-line tool to predict the tenderness of each carcass and as a tool to predict the tenderness potential from a biopsy sample of a live animal).

A procedure to carry out ELISA's for both HSP 20 and 27 has been developed. This procedure is currently being refined and validated to establish the Standard Operating Procedure (SOP). The SOP will then be used to measure assay reproducibility, sensitivity and linear dynamic range. This work is currently underway and will be complete within the next two weeks. Once complete, the ELISA's will be used for routine screening of samples required as part of earlier milestones.

The protocol is as follows:

i) HSP20

1. An appropriate number of wells in a 96-well polystyrene plate with flat bottoms were coated with 5µg/ ml solution of mouse anti-HSP20 monoclonal antibody in carbonate buffer, pH 9.4. The plate was incubated overnight at 4°C. The excess antibody was then aspirated off.
2. The wells were blocked with a solution of casein in PBS for 60min at 37°C. The blocker solution was then aspirated off.
3. A 5ng/ ml solution of human HSP20 was added to the first row of the plate. This solution was diluted 2-fold from Rows A to G with blocker. Row H did not receive any HSP20 and served as the negative assay control. The plate was incubated for 60min at 37°C. The plate was then washed three times with PBS containing Tween20.
4. Each of the wells then received a 1 in 4000 dilution of goat anti-HSP20 polyclonal antibody prepared in blocker. The plate was incubated for 60min at 37°C. The plate was then washed three times with PBS containing Tween20.
5. Each of the wells then received a 1 in 4000 dilution of anti-rabbit antibody conjugated with horseradish peroxidase prepared in blocker. The plate was incubated for 60min at 37°C. The plate was then washed three times with PBS containing Tween20.
6. Amount of HSP20 was measured by incubating the plate with OPD solution containing hydrogen peroxide and the reaction was stopped by addition of sulfuric acid. The absorbance was measured at 490nm.
7. The intensity of the color was plotted as a function of HSP concentration.

ii) HSP27

1. An appropriate number of wells in a 96-well polystyrene plate with flat bottoms were coated with 5µg/ ml solution of mouse anti-HSP27 monoclonal antibody in phosphate-buffered saline. The plate was incubated overnight at 4°C. The excess antibody was then aspirated off.
2. The wells were blocked with a solution of casein in PBS for 60min at 37°C. The blocker solution was then aspirated off.
3. A 100ng/ ml solution of human HSP27 was added to the first Row of the plate. This solution was diluted 2-fold from Rows A to G with blocker. Row H did not receive any HSP27 and served as the negative assay control. The plate was incubated for 60min at 37°C. The plate was then washed three times with PBS containing Tween20.

4. Each of the wells then received a 1 in 1000 dilution of goat anti-HSP27 polyclonal antibody prepared in blocker. The plate was incubated for 60min at 37°C. The plate was then washed three times with PBS containing Tween20.
5. Each of the wells then received a 1 in 4000 dilution of anti-rabbit antibody conjugated with horseradish peroxidase prepared in blocker. The plate was incubated for 60min at 37°C. The plate was then washed three times with PBS containing Tween20.
6. Amount of HSP27 was measured by incubating the plate with OPD solution containing hydrogen peroxide and the reaction was stopped by addition of sulfuric acid. The absorbance was measured at 490nm.
7. The intensity of the color was plotted as a function of HSP concentration.

4.4 Report on interaction of low molecular weight HSP with post mortem tenderisation and meat quality

Heat shock proteins (HSP) 20 and 27 are inducible chaperone proteins that are produced by cells in response to metabolic and environmental changes. Our previous milestone confirmed the observations that the activity of the calpain enzyme system, as measured by the calpain fluorescence assay, was correlated with the measured concentrations of HSP. The original experiments demonstrated that HSP 20 and 27 are bound to both calpains and calpastatin, as determined by immunoprecipitation experiments, the implication therefore is that these low molecular weight chaperone proteins can influence meat quality.

The objectives of this milestone were to develop further the role of HSPs on post mortem tenderisation and possible influences on other meat quality attributes.

15°C Pre-rigor temperature treatment:
These experiments confirm the previous studies showing a relationship between low molecular weight HSPs and aspects of tenderness. In particular, HSP concentrations were positively correlated with final shear force values ($r=0.61$; $P<0.001$), but less so with initial shear force values ($r=0.35$; $P<0.05$) and was not significantly correlated with ageing rates (See Figure 10).

HSP concentrations were not correlated with WBC or meat colour attributes of samples incubated at 15°C

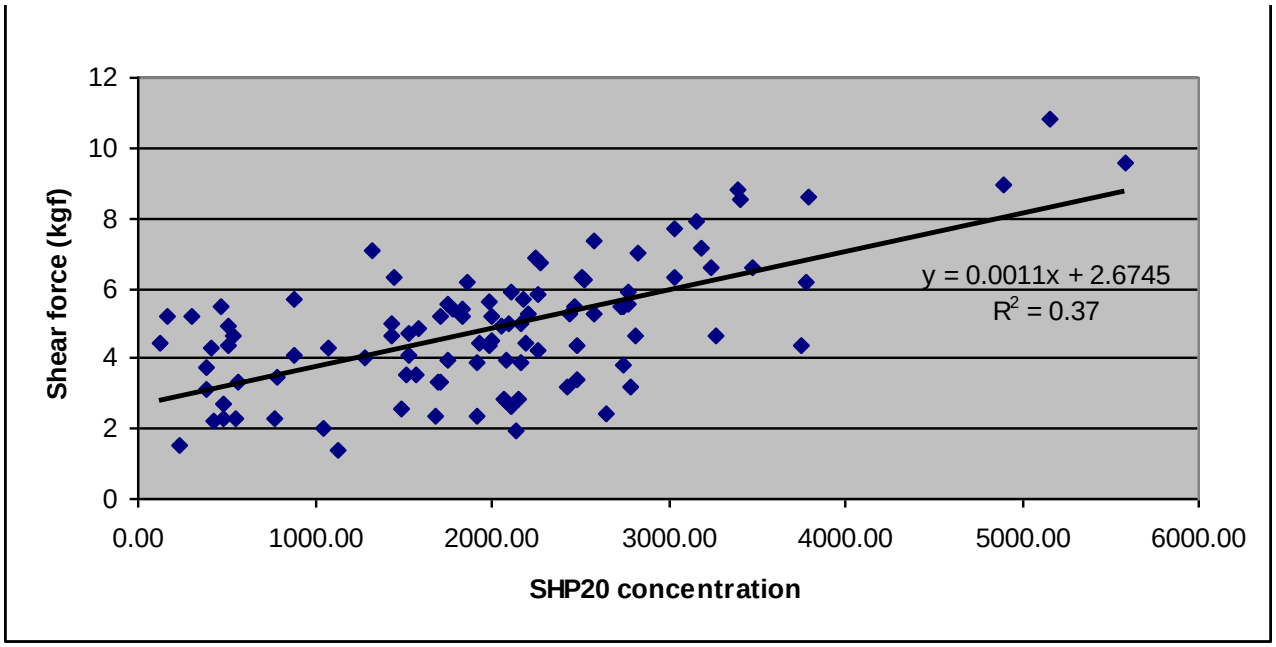

Figure 10: Relationship between HSP20 expression and final tenderness.

40°C Pre-rigor temperature treatment:
The sample size for the 40°C incubated samples is significantly smaller but still offers good indicators of interactions between HSP and meat attributes in muscles subjected to denaturing processing conditions.

In contrast to the 15°C samples, the tenderness parameters were not affected by HSP concentrations. However, HSP27 was significantly and positively correlated with both WBC (r^2 =0.44; $p<0.005$) and unbloomed colour (hue; r^2 =4.7; $p<0.005$). A similar trend was evident with HSP20, but was less marked compared with HSP 27.

These results confirm a relationship between the intramuscular concentration of small molecular weight HSPs and tenderness, presumably through their effects on calpain activity. This relationship was lost when the muscles were incubated under denaturing conditions during the pre-rigor period, which probably reflects more rapid loss of calpain activity caused by these high temperatures. In spite of this, there remains a significant relationship between ultimate tenderness from the same samples incubated at either 15° or 40°C; however, the 40°C maintained samples were 3.1 kgf higher than the 15°C samples.

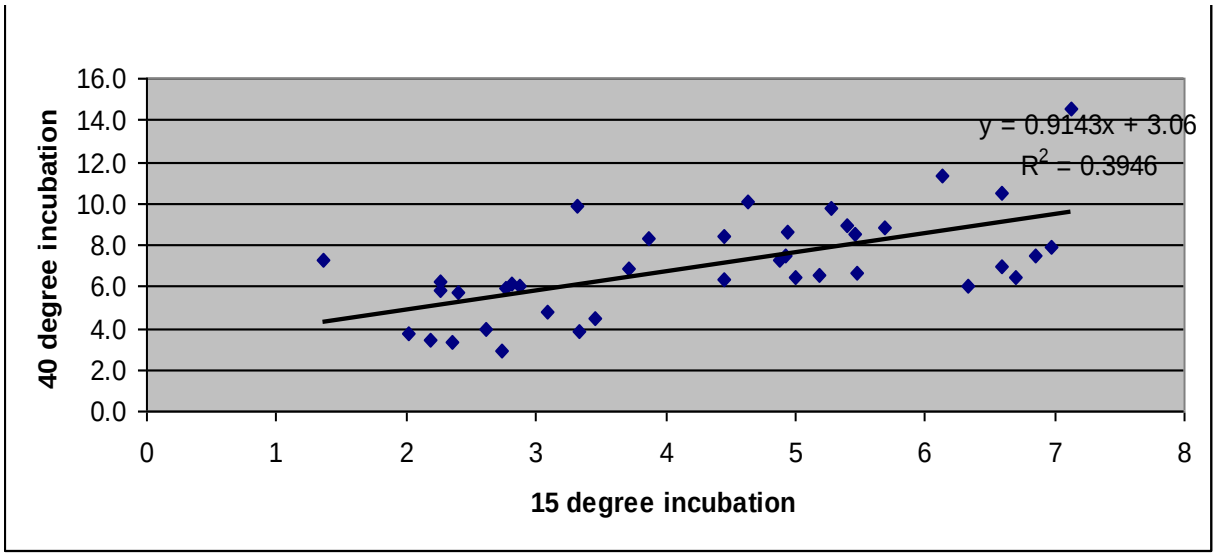

Figure 11: Final shear force values: comparing pre-rigor incubation at 15'C vs 40'C.

The evident influence of HSP concentrations on WBC and colour of samples at 40°C probably reflects a role on protein denaturation, since one role of chaperone proteins is to stabilise associated proteins under adverse environmental conditions. Increased WBC is normally associated with less myofibrillar lattice shrinkage, so it could be inferred that HSPs reduce myosin denaturation. Less myofibril denaturation would also be expected to reduce reflectance and, hence, reduce L* values, but this expectation was not confirmed. However, the change in Hue value in unbloomed meat may identify an influence of HSP on myofibrillar structure.

4.5 Using HSP's as a quantitative measure provide a preliminary report outlining some of the best practice methods for stock presentation at point of slaughter

A clear variation in the HSP20 and 27expression is evident in the sample population (Figure 12). The mean values for the HSP expression according to the preslaughter treatment is shown in Table 1.

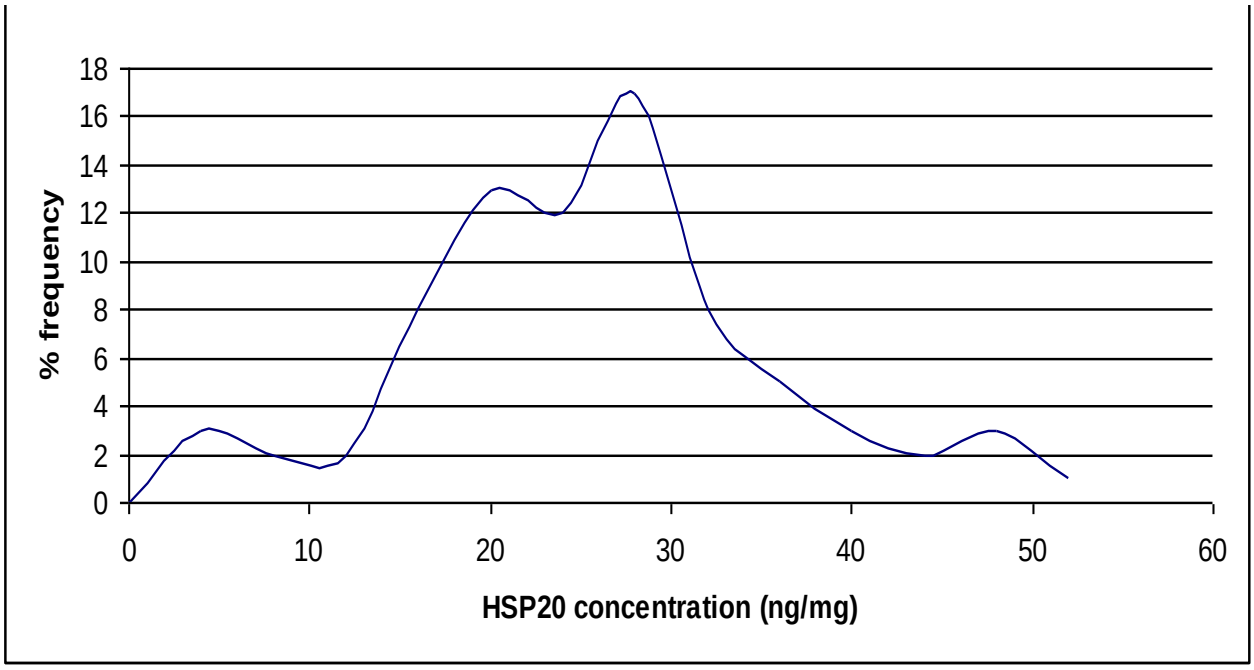

Figure 12: Frequency distribution of HSP20.

The HSP20 expression was lowest in the consignments from local Waikato farms. In contrast, significantly higher levels ($P<0.01$) were found in the consignments that were subjected to overnight transport over several hours. The three sample groups from local sale yards did not differ significantly from cattle delivered directly from local farms although these values tended to be slightly higher. Interestingly, the variation in the HSP's between animals tended to be far greater when they had been subjected to sale yards or overnight long-distance transport compared to those that arrived at the plant direct from local farms. Although the results of HSP27 showed similar trends, the differences were not significant.

Table 1: Results of pre-slaughter treatments on HSP20 and HSP27 expression

Consignment source	HSP20 ng/mg (SEM)	HSP27 absorbance units (SEM)
Local farm	21.0[a] (2.48)	2484.8 (534.7)
Overnight transport	36.91[b] (5.64)	3174 (1253.4)
Local sale yard	27.29[a] (5.64)	2497.1 (834.7)
Significance	**	ns

Note: Values with different superscripts within a row are significantly different

The ELISA assay for HSP20 proved to be rapid and highly reproducible. The experience with HSP27 was disappointing and, at this stage, the problem seems to be the source of the antibody: although the same antibody is used successfully for the western blot analysis and,

therefore, binds effectively with bovine HSP27, the quantities needed in the ELISA are far less than is the case for the HSP20, and this may reflect a loss of reactivity in the condition of the ELISA. An alternative source for the HSP27 antibody has been found and the new antibody will be evaluated over the next few weeks.

Both assays demonstrate a significant range of HSP response in the post mortem muscle. This identifies that there are considerable individual differences in the induced expression of the HSP proteins and this is particularly marked when they have been subjected to potentially stressful pre-slaughter treatments. Intuitively, the short transport distances and rapid slaughter of cattle produced local to the slaughter plant would be expected to show the lowest physiological responses during the pre-slaughter experience, and this expectation is confirmed in the HSP20 measurements. Increasing the travel distances to require overnight transport produced a significant increase in HSP20 expression. Perhaps surprisingly, the sale yards had little effect on HSP expression. However, the sale yards were local to the slaughter-plant and the cattle were also produced locally and travelled only limited distances. One implication is that the HSP20 responds to physical exertion, rather than any emotional stress associated with the novel environment.

5 Impact of this Research

The implication of this research is that the modified calpain assay offers some important insights into the determinants of tenderness attributes. Of particular interest is the importance of the susceptibility assay, which would seem to suggest that the state of the protein substrate has an important role in the rate of tenderisation, rather than simply the state of the enzyme system. A confirmation of the HSP measurements using the ELISA assay will be included in the analyses to confirm these conclusions.

6 Conclusions

The current research found that the calpain fluorescence assay demonstrated effective tenderness prediction, and also interactions with the levels of heat shock proteins. Of particular interest were the results of the protein substrate susceptibility assay, which shows that the rate of tenderisation depends on the state of the structural proteins that are degraded by the calpain enzyme, and is not solely dependent on the enzyme activity itself.

A rapid assay procedure to carry out ELISA’s for both HSP 20 and 27 has been developed. This procedure is currently being refined and validated to establish the Standard Operating Procedure (SOP). The SOP will then be used to measure assay reproducibility, sensitivity and linear dynamic range. Once complete, the ELISA’s will be used for routine screening of samples.

The use of HSP to assess pre-slaughter stress and optimise pre-slaughter handling systems requires further research.

7 Recommendations

The use of HSP to assess pre-slaughter stress and optimise pre-slaughter handling systems is currently underway. Therefore, it is proposed that further work is required as follows :

- Develop muscle biopsy procedures to allow routine on-farm measurements of calpain and HSP activity.
- Identify production and pre-slaughter management contributions to 24 hour tenderness.
- Identify sources of variable substrate susceptibility to calpain degradation and tenderisation.