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Inhibition of protease activity. Part 1. The effect on tenderness and indicators of proteolysis in ovine muscle

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Abstract

To examine the effect of particular enzyme groups on tenderness specific cysteine protease inhibitors were injected into muscle early post-mortem. The protease enzyme inhibitor E-64 was injected into the *m. longissimus thoracis et lumborum* (LTL) on the right side of 12 lamb carcasses within 15 min of death and in another 12 carcasses with the protease inhibitor Z-Phe-Ala-CHN_2. The left LTL (control) was injected with saline (0.25 M NaCl). To create variation in the rate of pH decline alternate carcasses were electrically stimulated (low voltage). The LTL was divided into cranial and caudal portions and aged for 1 or 2 days. Muscle samples at 1 day post-mortem were used for measurement of osmolality and sarcomere length ($n=48$), and others at 1 and 2 days post-mortem for shear force determination ($n=96$). The myofibrillar fragmentation index (MFI) was determined on samples taken at pH 6.2 and 1 and 2 days post-mortem ($n=144$). Other muscle samples were obtained at death, pH 6.2 and 6.0 and then at 1 and 2 days post-mortem ($n=215$). These samples were used for determination of protein solubility and the concentration of free amino acids. Stimulation caused a faster ($P<0.05$) decline in pH. There was no effect of stimulation ($P>0.05$) on shear force values, but injection of inhibitor and ageing both had effects ($P<0.001$). The inhibitor E-64 prevented any improvement in tenderness with ageing, whereas the inhibitor Z-Phe-Ala-CHN_2 and the control samples showed a similar ageing response. In the latter two treatments there was an average reduction of 1 kg in shear between 1 and 2 days post-mortem, whilst the inhibitor E-64 maintained shear force on average 2 kg higher than control samples. Injection and ageing had an effect on MFI ($P<0.001$) and there was an interaction ($P<0.05$) between stimulation and ageing for MFI, such that as stimulated muscle aged the rate of change of MFI was greater. There was an interaction between injection and ageing ($P<0.05$) for protein solubility such that samples treated with E-64 showed a minimal increase in protein solubility with ageing, whereas in samples treated with Z-Phe-Ala-CHN_2 and the control samples there was a significant increase. There was also an interaction between stimulation and ageing such that between sampling at pH 6.0 and 2 days post-mortem, stimulated muscle exhibited greater solubility ($P<0.05$). There were no effects ($P>0.05$) on the concentration of free amino acids. The evidence indicated that the cysteine proteases were responsible for post-mortem proteolysis and tenderisation, in particular the calpains, whereas the cathepsins (B and L) were unlikely to contribute to proteolysis and subsequent tenderisation in meat.

Keywords: Inhibitors; Proteases; Tenderness; Proteolysis; Lamb

1. Introduction

There is evidence that the cysteine proteases, in particular the calpains, are responsible for myofibrillar protein degradation (Uytterhaegen, Claeys, & Demeyer, 1994). However there is some debate about the role of the other major group of cysteine proteases comprising several of the cathepsins. Ouali (1992) reviewed mechanisms controlling post-mortem tenderisation and cited results from experiments using both rabbit and beef muscles, which suggested that the cathepsins did contribute to degradation. However, Koohmaraie, Whipple, Kretchmar, Crouse, and Mersmann (1991) showed that although lamb and beef muscle had similar cathepsin B and B + L activity measured at death, the rate of tenderisation in the different species was very different. From this they inferred that cathepsins made little contribution to the degradation of myofibrillar proteins. By contrast, O'Halloran, Troy, Buckley, and Reville (1997) suggested that the higher activity of

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cathepsins B and B + L in the soluble fraction from fast glycolysing muscle was in part responsible for a lower shear force measured at 2 and 6 days post-mortem, compared with slow glycolysing muscle.

As an alternative approach, there have been few studies in which inhibitors to the cysteine proteases have been used in situ to quantify their contribution to post-mortem tenderisation. Under an in vitro system Sugita, Ishiura, Suzuki, and Imahori (1980) showed that E-64 (trans-Epoxysuccinyl-L-Leucylamido (4-Guanidino)-Butane) inactivated the calpains, while Barrett et al. (1982) reported that this compound also inhibited the cathepsins B, H and L. In a previous study, we clearly demonstrated that injection of E-64 into the muscle pre-rigor significantly reduced tenderisation (Hopkins & Thompson, 2001). Using a thorough method of injecting inhibitors into muscle, Uytterhaegen et al. (1994) found that a range of inhibitors, including E-64, injected at 1-day post-mortem effectively stopped the ageing process. Uytterhaegen et al. (1994) also injected other inhibitors selective for the cathepsins B, L, D and H and suggested that their contribution to post-mortem tenderisation was minimal. Although this provided strong evidence for the role of the calpains in meat tenderisation, Dransfield (1999) concluded, that the role of the calpains and cathepsins in tenderisation had not been clarified by the use of inhibitors due to conflicting results.

This paper examined the effect of cysteine proteases on tenderness during the early post-mortem period using muscle that was injected in situ with protease inhibitors targeted at specific enzyme groups. Low voltage stimulation was used to vary the rate of glycolysis as a means of altering the pH/temperature relationship as muscle entered rigor. This tool was used because both pH (rate of decline) and temperature have been shown to affect calpain activity (McDonagh, 1998; Simmons, Singh, Dobbie, & Devine, 1996). A subsequent paper will describe the effect of these inhibitors on degradation of specific proteins and myofibril and sarcomere integrity and also examine the increase of free calcium in the sarcoplasm. A preliminary report of some aspects of the results relevant to this paper has been presented Hopkins, Littlefield, and Thompson (2000b).

2. Materials and methods

2.1. Experimental design

The experimental design was a 2×3 factorial with alternate carcasses electrically stimulated (low voltage) and within each carcass the right side was injected with one of two protease inhibitors and the left side with saline (control). Muscle samples were taken at death and within each side at four other times up to 2 days post-mortem. These samples were used for a range of assays to follow changes in protein structure and larger samples were taken at 1 and 2 days post-mortem to determine shear force.

2.2. Animals and slaughter procedures

The experiment used 24 castrated male, second cross (Poll Dorset×Border (= one breed) Leicester×Merino) lambs, approximately eight months of age. The lambs were slaughtered in groups of eight over a 3-week period. Prior to slaughter each group of eight lambs were housed on a slatted floor and fed wheat based concentrate pellets along with lucerne hay, lucerne chaff and pasture hay for 2 weeks. At slaughter lambs were taken from the animal house and transported a short distance to the research abattoir where they were killed by use of a captive bolt and exsanguination. Two companion lambs were kept with the experimental lambs during the pre-slaughter feeding period and at slaughter, in an effort to reduce stress amongst the slaughtered lambs. Standard dressing procedures were followed.

2.3. At-death muscle sampling and stimulation

Immediately after slaughter and dressing a muscle sample of approximately 12 g was taken from a portion of the *m. longmissius thorasis et lumborum* (LTL) as shown in Fig. 1. Muscle samples weighing 2 ($n=4$), 1 ($n=1$) and 3 g ($n=1$) were frozen in liquid nitrogen and kept frozen at -70°C. The 2-g samples were kept for SDS electrophoresis, protein solubility and free calcium determination and the 3-g samples for determination of free amino acids with results for the assays (SDS electrophoresis and free calcium) to be reported in the following paper. The 1-g samples were used for measurement of pH using the iodoacetate procedure. Immediately after sampling, alternate carcasses (four per slaughter) were stimulated using a low voltage system for 40 s (45 V, with 36 pulses per s and a pulse width of 25 ms; H.E. Technologies, Qld, Australia). Injection of the selected muscle portions immediately followed stimulation.

2.4. Injection procedure

The protease enzyme inhibitor E-64 (trans-Epoxsuccinyl-L-Leucylamindo-(4-Guandino) Butane (Sigma-Aldrich Corporation, MO, USA) was injected into the LTL on the right side of 12 carcasses (four per slaughter) within 15 min of death, firstly between the 10th and 12th rib and then between the first lumbar vertebrae and the chump (Fig. 1). To ensure coverage 50 ml of solution was dispersed per loin, the lower portion received four injections (20×4) and the upper 13 injections

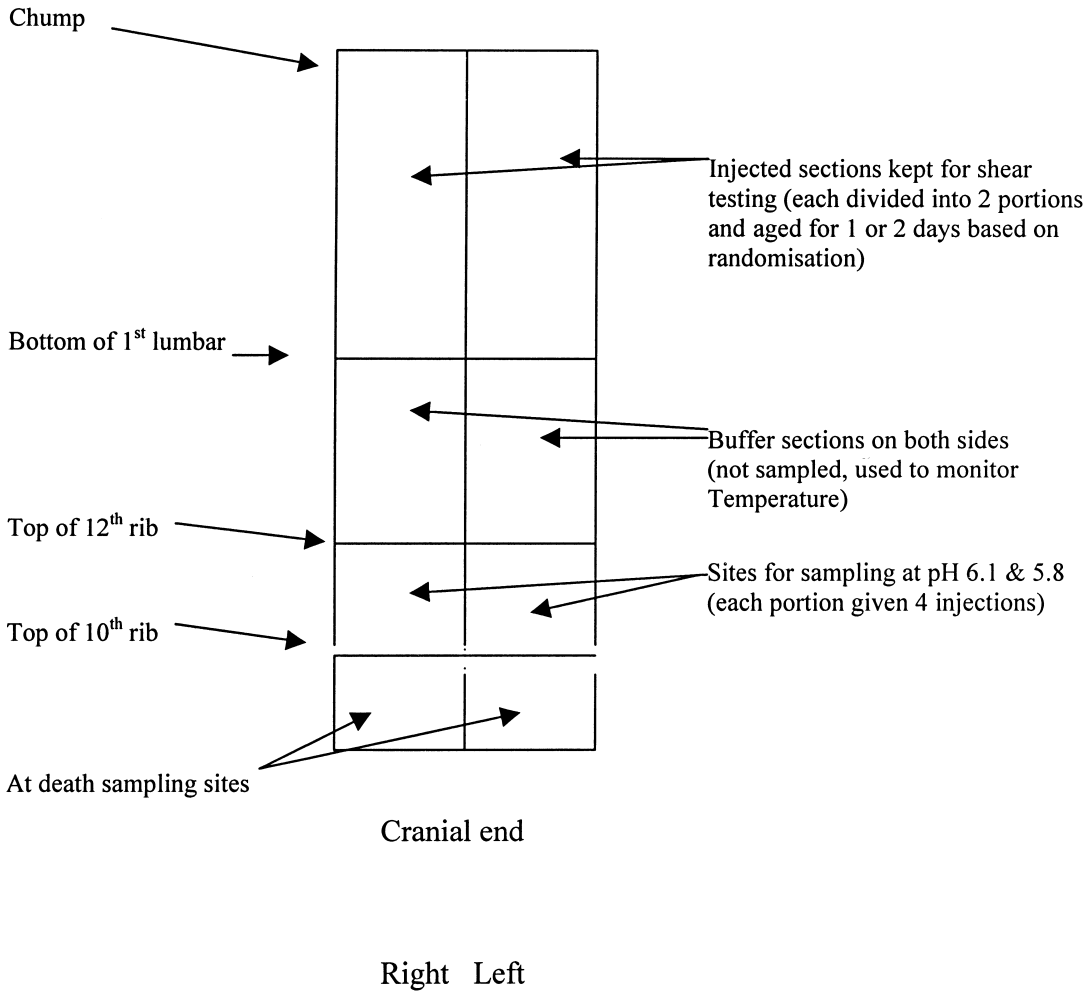

Fig. 1. Diagrammatic illustration of the sampling sites for the *m. longissimus thoracis et lumborum*.

(20×13 injection sites) using a bank of 20 18-gauge needles. Delivery of the solutions was controlled using a multiple syringe pump, which delivered 25 mg of E-64 (1.4 mM) per loin made up in a solution of saline (0.25 M NaCl) containing 20% dimethyl sulfoxide (DMSO). Injection always progressed in a caudal direction for the upper portion of the loin.

In the remaining 12 carcasses (four per slaughter) the protease inhibitor Z-Phe-Ala-CHN_2 (N-carbobenzoxy-phenylalanyl-alanyl-diazomethylketone; BACHEM AG, Baubendorf, Switzerland) was injected at 4.27 mg per loin (0.2 mM) as used by Uytterhaegen et al. (1994), using the same procedure as for the E-64 inhibitor. DSMO was used to dissolve the Z-Phe-Ala-CHN_2 (Kirschke & Shaw, 1981). The saline concentration used was designed to maintain equivalent conductivity to an isotonic saline solution in water. The left LTL (control) of each carcass ($n=24$) was injected with 50 ml of saline (0.25 M NaCl) containing 20% DMSO at the same time as the right side was injected. The same procedure was used to inject the saline, but with a separate injecting unit.

2.5. Chilling, pH measurement and muscle sampling

The carcasses were chilled at 6°C for 17–22 h. Temperature decline was determined using Cox recorders (Belmont, NC, USA), with probes inserted into the *m. semimembranosus* (SM) to the depth of the femur) and the center of the LTL (location as shown in Fig. 1).

pH was measured in the left-hand section of the LTL from which the at death muscle samples were taken (Fig. 1), at regular intervals using an Orion meter (Model 250 A, Orion Research International, MA, USA) with an Ionode IJ 42 electrode. When pH reached 6.1, muscle samples (2 g, $n=4$; 1 g, $n=1$; 3 g, $n=1$) were taken from each portion of the LTL as shown in Fig. 1 and prepared in an identical fashion to those taken at death. For one lamb, samples taken from the right side at this sampling were misplaced. The sampling procedure was repeated when the pH reached 5.8. Muscle samples (1 g) held at −70°C were used for determination of pH using iodoacetate. The method was adapted from that described by Dransfield, Etherington, and Taylor (1992). Frozen muscle samples were homogenised

using an Ultra Turrax at 25,000 rpm in 6 ml of cold buffer. The buffer contained 5 mM iodoacetate and 150 mM of KCl adjusted to pH 7 with KOH at 4–5°C. Samples were homogenised for three bursts of 15 s with breaks of 30-s on ice. The suspensions were then incubated in a waterbath at 20°C and the pH measured using an Orion meter (Model 250 A, Orion Research International, MA, USA) with an Ionode IJ 42 electrode which was calibrated at 20°C. The mean pHs of samples taken at pH 6.1 and 5.8 were shown to be 6.2 and 6.0 using the iodoacetate procedure and therefore the latter pH values will be used in subsequent discussion.

2.6. Sample preparation for objective and biochemical measurements

At the pH 6.0 sampling a 4–5 g sample of LTL was taken for subsequent determination of myofibrillar fragmentation and frozen at −20°C. After the chilling period the cold carcass weight was recorded and fat depth over the 12th rib measured and the injected portions of the LTL between the first lumbar and the chump removed from both sides of the carcass.

Injected sections of the LTL from both sides of the carcass as shown in Fig. 1 were divided into two portions and randomly assigned to ageing periods of 1 or 2 days. Portions to be aged were vacuum packed and held at 2–3°C. For day 1 portions, samples were frozen at −20°C for measurement of osmolality, sarcomere length and myofibrillar fragmentation index (MFI) determination. Samples for the latter two measures were taken from the lateral sides of the muscle before the 65-g cooking block was prepared. This was necessary to ensure subsequent separation of fibres along the direction of the myofibrils. Subcutaneous fat and the epimysium were removed for preparation of the 65-g block, which was then stored at −20°C until cooking and subsequent shear testing. Muscle tissue weighing 2 ($n=4$), 1 ($n=1$) and 3 ($n=1$) was also prepared from each portion and frozen in liquid nitrogen and kept frozen at −70°C as for pre-rigor samples. This same sampling procedure was undertaken on portions aged for 2 days, but samples for measurement of osmolality and sarcomere length were not kept.

2.7. Determination of cathepsin inhibitor efficacy

A test of the efficacy of the inhibitor Z-Phe-Ala-CHN_2, to inhibit cathepsins B and L was undertaken. Sub-samples (2 g) of muscle were removed from a section of LTL, which had been aged for 1 day post-mortem prior to freezing. The muscle was thawed and chilled at 4°C for 2 days. All subsequent steps were carried out at 1–4°C, or on ice. Approximately 1.0 g of muscle tissue, removed of all visible connective tissue, was finely minced in 2.0 ml of buffer (100 mM sucrose, 100 mM KCl, 50 mM Tris–HCl, 10 mM $Na_2H_2P_2O_7$ and 1 mM Na_2EDTA, pH 7.2) containing 50 μg of nargarse (Protease Type XXVII, Sigma-Aldrich Corporation, MO, USA) dissolved immediately prior to use, as used by O'Halloran et al. (1997). The minced muscle sample was incubated for 5 min in the buffer + nagarse solution, the excess buffer was decanted and the mince homogenised in 20 ml of buffer without nagarse. A motorised Potter-Elvehjem homogeniser with glass attachments was used. Homogenisation involved seven passes at 1000 rpm after which the homogenate was filtered through cheesecloth.

The cathepsin B + L activity assay utilised the substrate Z-Phe-Arg-NMec (N-CBZ-L phenylalanyl — L-arginine-7-amido-4-methylcoumarin, Sigma- Aldrich Corporation, MO, USA) common to both cathepsin B and cathepsin L. A range of concentrations (0, 0.1, 0.2, 1.0, 5.0, and 10.0 mM) of the cathepsin inhibitor Z-Phe-Ala-CHN_2 were included in the assay. The assay consisted of 50 μl of muscle homogenate plus 50 μl of inhibitor plus 250 μl of assay buffer (250 mM sodium acetate, 2.5 mM Na_2EDTA, 0.05% Brij 35 and 5 mM dithiothreitol, added freshly, pH 5.5). This was incubated for 2 min at 40°C. After the addition of 200 μl of substrate (10 mM Z-Phe-Arg-NMec in DMSO stored at 4°C and diluted to 12.5 μM with deionised water prior to the assay) the reaction was incubated at 20, 30 or 37°C for 30 min.

The cathepsin B + L assay reactions were halted by the addition of 2.5 ml of stop solution (100 mM monochloroacetic acid in 100 mM sodium acetate, pH 4.3). Release of 7-amino-4-methylcoumarian (NH_2Mec) was measured fluorimetrically in triplicate for each sample with a Perkin-Elmer 1000 fluorescence spectrometer (Perkin-Elmer Ltd, UK). The wavelength for excitation was 360 nm and emission was measured at 440 nm (1000 emission units were equivalent to 0.5 μM of NH_2Mec). The response of the cathespin inhibitor in vitro, was influenced by the incubation temperature, but nevertheless even at a inhibitor concentration of 0.1 mM there was little activity compared to control samples (ie those with no inhibitor present), and as such this verified that the inhibitor was effective at 0.2 mM, the concentration used in the experiment. At higher concentrations of the inhibitor (5 and 10 mM) there was no activity of the cathepsins.

2.8. Measurement of shear force, sarcomere length, osmolality and myofibrillar fragmentation index (MFI)

The 65-g LTL samples were cooked from the frozen state for 35 min in plastic bags at 70°C in an 80-l waterbath for determination of shear force as described by Hopkins and Thompson (2001). Determination of sarcomere length and osmolality on 1 day post-mortem samples was as described by Hopkins and Thompson

(2001). A thin slice of frozen muscle from each portion of the LTL was used for determination of MFIs as described by Hopkins, Littlefield, and Thompson (2000a).

2.9. Protein solubility

Protein solubility was determined on muscle samples obtained at death, pH 6.2 and 6.0 and then at 1 and 2 days post-mortem ($n=215$). The method was adapted from that described by Uytterhaegen et al. (1994). Samples of muscle (2 g) held at −70°C were homogenised using an Ultra Turrax at 25,000 rpm with 15 ml of cold extraction buffer for three bursts of 15 s with 30-s rests on ice. Five millilitres of buffer was used to wash down the shaft. The extraction buffer was 0.1 M KCl, 1 mM EGTA, 1 mM NaN_3, 1 mM $MgCl_2$ and 28 mM potassium phosphate (8 mM KH_2PO_4, 20 mM K_2HPO_4, pH 7.0 at 4–5°C). The suspension was centrifuged at 1500 *g* for 10 min and the pellet was homogenised again using the same regime as the initial homogenisation. After a further 10 min centrifugation at 1500 *g* the supernatant was discarded and 30 ml of the buffer was added (0.4 M NaCl, 89 mM $Na_2HPO_4.7H_2O$ [di-sodium hydrogen orthophosphate (heptahydrate)], 19 mM KH_2PO_4, 1 mM EDTA, 1 mM NaN_3, pH 7.0) to the isolated myofibrils. Suspensions were kept at 20°C in a temperature controlled room and gently agitated for 3 h on a roller mixer. The suspensions were then centrifuged at 3000 *g* for 30 min at 20°C and the supernatant decanted and kept frozen at −20°C until protein determination. Aliquots of protein from the supernatant were thawed and the protein concentration measured in triplicate using the BCA method (Pierce Chemical Company, IL, USA). Absorption was measured at 570 nm in microwells using a plate reader (Titertek Multiskan® Plus, Lab Systems OY, Helsinki, Finland) in accordance with the specified protocol and a BSA standard curve was used.

2.10. Free amino acids

Alpha-amino nitrogen (αNH_2–N) was determined as an index of total free amino acids on muscle samples obtained at death, pH 6.2 and 6.0 and then at 1 and 2 days post-mortem ($n=215$). Muscle samples (3 g) held at −70°C were homogenised in 10 ml of deionised water with an Ultra Turrax at 25,000 rpm for three bursts of 15 s with 30-s breaks on ice. The shaft was washed down with 5 ml of deionised water. Fifteen ml of cold 5% 5-sulfosalicylic acid dihydrate (SSA) was added to the homogenate and the suspension centrifuged (2800 *g*) at 5°C for 30 min. The supernatant was then filtered through a mesh grid (1.0 mm) and kept frozen at −20°C until testing. Prior to determination of alpha-amino nitrogen, the samples were thawed and centrifuged at 1000 *g* for 5 min. Aliquots (0.2 ml) of the supernatant were diluted with 0.8 ml of a 2.5% SSA solution. Samples were tested in duplicate using an autoanalyser (Techicon Equipment Pty, Ltd, Sydney, Australia) and the methodology described by Oddy (1974) with the following modifications. Absorbance was measured at 570 nm using a spectrophotometer (Model UV-1201, Shimadzu Corporation, Japan) with the flow cell linked to the autoanalyser. A different wavelength was used to Oddy (1974), because Blackburn (1968) clearly showed that maximum absorbance occurs at 570 nm. Also standards were measured at the start of every day and after every 40 test samples. Repeat tests were performed on all samples that exceeded the overall mean value for all samples by two standard deviations. In this case, frozen samples (−70°C) were used after being prepared as per the method.

2.11. Statistical analysis

2.11.1. Non-linear model (pH decline)

The rate of pH decline relative to time post-mortem for each carcass side was described using data for the five different sample points, including the 'at-death' sample (which was common to both sides) using the following non-linear equation and a non-linear procedure SAS (1997):

$$\mathrm{pH}_t = \mathrm{pH}_\mathrm{f} + \left(\mathrm{pH}_\mathrm{i} - \mathrm{pH}_f\right)\exp^{-kt} \quad (1)$$

Where pH_t = pH at time t, pH_f = the ultimate pH, pH_i = the pH at $t=0$, k = rate constant of pH decline and t = the time in hours.

Estimates of k for Eq. (1) were derived for each carcass and this parameter was then analysed using a mixed model procedure which contained fixed effects for stimulation and injection and the interaction term stimulation×injection, with animal nested within stimulation as a random term.

2.12. Non-linear model (pH and temperature)

To determine the relationship between pH and temperature, pH data were averaged over all carcasses within sampling times and within stimulation treatments and the relationship between pH and temperature was derived using the following non-linear equation and a non-linear procedure SAS (1997):

$$\mathrm{pH}_x = \mathrm{pH}_\mathrm{f} + \mathrm{pH}_\mathrm{i} x^k \quad (2)$$

Where pH_x = pH at temperature x, pH_f = pH when x = zero, pH_i = the pH at the highest temperature, x = the temperature in degrees and k = rate constant of pH decline. Estimates of k were derived for the two groupings of carcasses (stimulated and non-stimulated).

2.13. Linear model (objective measures)

Shear force measurements, sarcomere length, osmolality, MFI, protein solubility, and the concentration of free amino acids were analysed using a mixed model procedure (SAS, 1997) which contained fixed effects for stimulation (stimulated or control), injection (inhibitor A, B or control) and post-mortem age of sample (pH 6.2, pH 6.0, 1 or 2 days post-mortem), all first order interactions and a random term (animal nested within stimulation). As osmolality and sarcomere length were only measured on 1-day samples, post-mortem age was not included in the model. Non-significant interactions ($P > 0.05$) were removed from the model until the final model for each dependent variable was obtained. Predicted means were compared using the PDIFF statement, which enables multiple comparisons.

For shear force data the effect of adding the covariates pHk [from Eq (1)], or MFI was tested (with the stimulation term dropped from the model when the effect of pHk was tested). In a similar manner the covariate pHk [from Eq. (1)] was tested against MFI, protein solubility and the concentration of free amino acids data, in which case the stimulation term was again dropped from the model. Using data for samples at 1-day post-mortem the effect of osmolality as a covariate on shear force and MFI data was also tested.

3. Results

3.1. Carcass characteristics and pH decline during chilling

The mean ($\pm$S.D.) carcass weight and 12th rib fat depth was 26.2 ± 1.6 kg and 4.0 ± 1.5 mm, respectively. Stimulation caused a faster decline ($P < 0.05$) in pH (k values of 0.26 and 0.11 for stimulated and non-stimulated carcasses, respectively). However there was no effect of injection, or the interaction term injection$\times$stimulation, on k values. The decline in pH (k) with time post-mortem was significant ($P < 0.001$).

Overall, stimulation resulted in higher temperatures ($P < 0.05$) at time of sampling than in non-stimulated carcasses (k values of 0.029 and 0.019 for stimulated and non-stimulated carcasses, respectively).

3.2. Objective meat quality measurements

Injection and ageing both had an effect ($P < 0.001$; Table 1) on shear force values. There was no effect ($P > 0.05$) of stimulation, but the interaction term injection$\times$ageing did have an effect ($P < 0.05$; Table 1). The F-ratios in Table 1 indicated that injection had the largest impact on shear force values, followed by ageing and the interaction between these two terms. The inhibitor E-64 prevented any improvement in tenderness with ageing, whereas the inhibitor Z-Phe-Ala-CHN_2 and the control samples showed a similar response. In the latter two treatments there was an average 1 kg improvement in shear force between 1 and 2 days post-mortem, whilst the inhibitor E-64 maintained shear force at an average of 2 kg higher than control samples. Including covariates for MFI, or pHk in the model for shear force had no effect ($P > 0.05$).

There was no effect of stimulation, or injection on sarcomere length, or osmolality ($P > 0.05$) and for this reason the results are not shown. Further to this, as a covariate, osmolality had no effect ($P > 0.05$) in models for shear force or MFI. For comparison with other published values osmolality values were 876, 858 and 864 mmol/kg for muscle injected with E-64, Z-Phe-Ala-CHN_2 and control muscle, respectively.

3.3. Indicators of proteolysis

Injection and ageing had an effect on MFI ($P < 0.001$), while stimulation had no effect on MFI, but the interaction term stimulation$\times$ageing did have an effect ($P < 0.05$; Table 2) on MFI. This showed that as stimulated muscle aged the rate of change of MFI was greater than for non-stimulated muscle. The F-ratios showed that ageing had the largest impact on MFI (Table 2). MFI explained 35% of the variation in shear force values overall ($P < 0.001$), but for samples injected with E-64 it only explained 10% of the variation. As a covariate, pHk had no significant effect on MFI.

For protein solubility, injection and ageing had an effect ($P < 0.001$), with ageing being the most important as indicated by the F-ratios (Table 3). There were significant interactions between injection and ageing ($P < 0.001$) and stimulation and ageing ($P < 0.05$). Stimulated muscle exhibited greater solubility between sampling at pH 6.0 and 2 days post-mortem than did control muscle, yet as a covariate pHk had no effect ($P > 0.05$). Protein solubility was unaffected by the inhibitors when measured at pH 6.0 and 6.2, but there was a large increase in solubility as the control and Z-Phe-Ala-CHN_2 treated samples aged thereafter, with a much smaller increase in samples treated with E-64. The effect of the inhibitor can be seen in Fig. 2a with solubility showing minimal change as post-mortem time progressed. The pattern in Fig. 2a for the control samples shows a significant increase in protein solubility as the muscle aged post-rigor. As shown in Fig. 2b, protein solubility in the Z-Phe-Ala-CHN_2 treated samples was similar to the control samples. There were no significant effects on the concentration of free amino acids and there was no clear pattern in the concentration of free amino acids as the pH declined post-mortem and no attempt was made to model the data.

4. Discussion

4.1. Rate of glycolysis

Low voltage (LV) stimulation of ovine carcasses clearly increased the rate of pH fall as expected (Chrystall, Devine, Ellery, & Wade, 1984; Hopkins & Ferrier, 2000). The rationale for including this treatment was based on the finding that calpastatin and μ-calpain activities have been shown strongly influenced by the rate of pH decline (McDonagh, 1998). This strategy therefore offered a method for potentially creating differences in the rate of proteolysis, allowing examination of the interaction between inhibitors and proteolysis rate.

4.2. Effect of the inhibitor E-64

The results from this study and Hopkins and Thompson (2001) showed that E-64 was effective at preventing tenderisation. In both studies, E-64 was administered soon after death, when temperature, pH and the moisture binding ability of the proteins were all high. Despite this Uytterhaegen et al. (1994) reported

Table 1
Predicted means (S.E.) of Warner-Bratzler shear values (WB; kg) from a model containing the terms for fixed effects and interactions as indicated, including a random term (animal nested within stimulation; $n = 96$)[a,b]

Terms	d.f.[c]	*F*-ratio	Level	WB		Average S.E.
Injection	2/67	65.6**	Inhibitor — E-64	7.2x		
			Inhibitor — Z-Phe-Ala-CHN_2	5.5y		
			Control	5.2y		0.25
Ageing	1/67	32.2**	1 (days)	6.3x		
			2	5.6y		0.23
Stimulation	1/22	1.5				
			Injection	Ageing		
				1 day	2 days	
Injection×ageing	2/67	5.0*	Inhibitor — E-64	7.3ax	7.1ax	
			Inhibitor — Z-Phe-Ala-CHN_2	6.0ay	5.0by	
			Control	5.7ay	4.6by	0.28

[a] Values followed by the same letter in a row (a, b) are not significantly different ($P > 0.05$).
[b] Values followed by the same letter in a column within a main or interaction term (x, y) are not significantly different ($P > 0.05$).
[c] d.f., Degrees of freedom (numerator/denominator).
*$P < 0.05$. **$P < 0.001$.

Table 2
Predicted means (S.E.) of myofibrillar fragmentation index (MFI) values from models containing the terms for fixed effects as indicated, including a random term (animal nested within stimulation)[a,b]

Terms	d.f.[c]	*F*-ratio	Levels	MFI		Average S.E.
Injection	2/114	14.9**	Inhibitor — E-64	67x		
			Inhibitor — Z-Phe-Ala-CHN_2	82y		
			Control	82y		2.4
Ageing	2/114	20.3**	pH 6.0	52x		
			1 day	72y		
			2 days	81z		2.2
Stimulation	1/22	0.8				
			Ageing	Stimulation		
				Stimulated	Control	
Ageing×stimulation	2/114	3.3*	pH 6.0	51ax	53ax	
			1 day	75ay	70ay	
			2 days	84az	77az	3.1

[a] Values followed by the same letter in a row (a, b) are not significantly different ($P > 0.05$).
[b] Values followed by the same letter in a column within a main or interaction term (x, y, z) are not significantly different ($P > 0.05$).
[c] d.f., degrees of freedom (numerator/denominator).
*$P < 0.05$. **$P < 0.001$.

Table 3
Predicted means (S.E.) for protein solubility (PS) per gram of muscle for samples taken at pH 6.2 and 6.0 and at 1 and 2 days post-mortem from models containing the terms for fixed effects and interactions as indicated, including a random term (animal nested within stimulation)[a,b]

Terms	d.f.[c]	*F*-ratio	Level	PS (mg/g)				Average S.E.
Injection	2/153	18.5**	Inhibitor — E-64	22.4x				
			Inhibitor — Z-Phe-Ala-CHN_2	30.8y				
			Control	31.2y				1.36
Ageing	3/153	98.0**	pH 6.2	19.1x				
			pH 6.0	18.6x				
			1 (days)	33.1x				
			2	41.8z				1.42
Stimulation	1/22	2.8						
			Injection	Ageing				
				pH 6.2	pH 6.0	1 day	2 days	
Injection×ageing	6/153	9.9**	Inhibitor — E-64	19.8ax	17.7ax	26.1bx	26.2bx	
			Inhibitor — Z-Phe-Ala-CHN_2	18.8ax	17.8ax	37.6by	49.1cy	
			Control	18.7ax	20.2ax	35.6by	50.1cy	2.18
			Stimulation					
Stimulation×ageing	3/153	5.1*	Stimulated	18.8ax	18.4ax	35.5bx	46.6cx	
			Control	19.4ax	18.8ax	30.7bx	37.0cy	1.97

[a] Values followed by the same letter in a row (a, b, c) are not significantly different ($P > 0.05$).
[b] Values followed by the same letter in a column within a main or interaction term (x, y, z) are not significantly different ($P > 0.05$).
[c] d.f., degrees of freedom (numerator/denominator).
*$P < 0.05$. **$P < 0.001$.

that when E-64 was injected at 1-day post-mortem there was still a significant reduction in tenderisation. The data presented here shows that E-64 decreased tenderisation on average by 38% compared with 57% in the report by Hopkins and Thompson (2001). Dransfield (1992) concluded that tenderisation due to the activity of calpains began when the pH fell to approximately 6.1, which if true implied that an inhibitor injected at 1 day post-mortem would not fully retard proteolysis. For this reason we opted to inject, at or near death, so the extent of proteolysis in the first 1–2 days post-mortem could be studied. These results are discussed in the second paper in this series.

The importance of inhibitor concentration was evident from the brief comparison of studies presented by Dransfield (1999). In addition delivery method of the inhibitor to the intracellular environment will impact on the response and it is argued that this perhaps explains why in the experiment of Aalhus, Dugan, and Best (1996), only small differences in shear force were reported between muscle injected with E-64 and control muscle. It could be argued that both these factors explain why ageing was not prevented when Alarcon-Rojo and Dransfield (1995) soaked *semitendinosus* muscle taken from bovine carcasses at 1 day post-mortem in a solution containing 1 μm E-64, at 10°C for up to 6 days. Results from our study and Uytterhaegen et al. (1994) suggest that the concentration used by Alarcon-Rojo and Dransfield (1995) was too low to be effective. Failure of the delivery methodology was also evident in the work of Etherington, Taylor, Wakefield, Cousins, and Dransfield (1990) where E-64 was injected into the bloodstream of chickens 1 hour before slaughter, but had no appreciable effect on the activity of selected cysteine proteases in muscle.

Of the few reports where E-64 has been used in muscle, the work reported here and previously (Hopkins & Thompson, 2001), is to our knowledge, the first to demonstrate the effect of E-64 injection, on a reduction in MFI values, with a reduction of 10% previously and 18% in this study. Additionally, this study showed a significant reduction (28%) in protein solubility in muscle injected with E-64, indicative of a reduction in proteolysis reinforcing why shear force was greater in muscle treated with E-64. The measurement of the combined concentration of free amino acids proved to be an insensitive indicator of myofibrillar proteolysis in the current study. Given that the concentration of some free amino acids fluctuates during ageing (Feidt, Petit, Bruas-Reignier, & Brun-Bellut, 1996) indices of total free amino acids will be less sensitive than measurement of individual amino acids. Also since measurement of amino acids is a secondary indicator of myofibrillar degradation and proteolysis of the sarcoplasmic proteins contributes to the pool (Toldrá, Flores, & Aristory, 1995) measurement of individual amino acids was not undertaken in this study.

The reduction in proteolysis and tenderisation of muscle injected with E-64 was consistent with inactivation of protease enzymes. There are now a number of studies, which have clarified the mode of inactivation. Varughese, Ahmed, Carey, Hasnain, Huber, and Storer

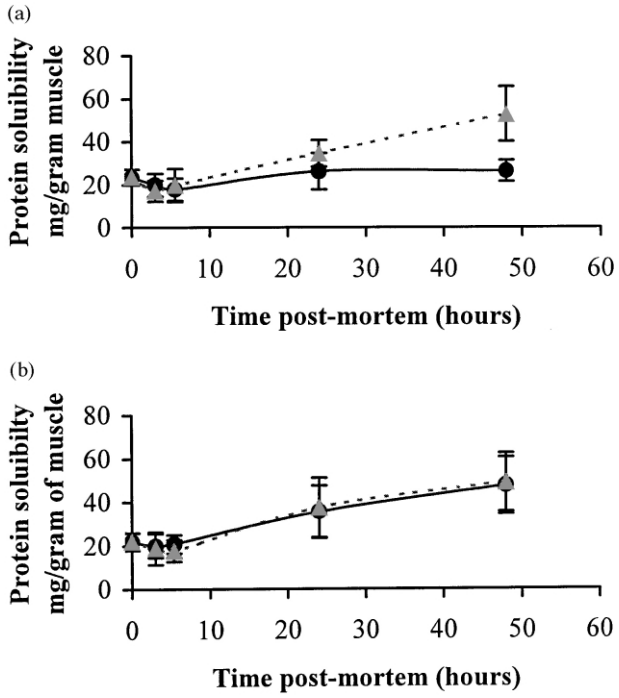

Fig. 2. (a) A plot of the mean (S.D.) protein solubility of muscle treated with E-64 (●—●) and control muscle (▲—▲) versus post-mortem time. (b) A plot of the mean (S.D.) protein solubility of muscle treated with Z-Phe-Ala-CHN_2 (●—●) and control muscle (▲—▲) versus post-mortem time.

(1989) reported that a covalent bond was formed between the thiol group of the cysteine 25 amino acid of papain and a carbon atom of E-64 which is consistent with the report of E-64 binding to the reactive thiol sites of cathepsin L (Fujishima, Imai, Nomura, Fujisawa, Yamamoto, & Sugawara, 1997).

4.3. Cysteine proteases

Results from the use of E-64 clearly show that the cysteine proteases were responsible for post-mortem proteolysis and tenderisation. However this inhibitor inactivates both the calpains (Parkes, Kembhavi, & Barrett, 1985; Sugita et al., 1980) and the cysteine cathepsins (Barrett et al., 1982) and so for this reason a specific inhibitor (a peptidyl-diazomethane) for the cathepsins B and L (Rich, 1986) was also used in the current study. Our results indicate very strongly that the calpains, are the causative enzymes involved in myofibrillar degradation and tenderisation, since the use of an inhibitor specific to cathepsins B and L did not result in differences in shear force, MFI values or protein solubility (Tables 1–3).

It could be argued that the injected cathepsin inhibitor would need to transverse the membrane of the lysosomes to be effective. However, for cathepsins to be effective at degrading myofibrillar proteins they must leak from the lysosomes (Zeece, Woods, Keen, & Reville, 1992), which is feasible given the failure of ion pumps in membranes during the development of rigor. Indeed there is some evidence that lysosomes do lose membrane integrity during ageing (Mobark, Connell, Reville, & Zeece, 1999; Zeece et al., 1992), but if this had occurred in the present study then the cathepsins would have been inactivated, given that the inhibitors must have reached the sarcoplasm as evidenced by the inhibitory effect of E-64. The other factor that must limit the activity of these enzymes outside of the lysosomes is the presence of the naturally occurring inhibitors, the cystatins (Zeece et al., 1992).

Although there is some evidence for correlations between cathepsin activity and tenderness (Ouali, 1992) there are a number of studies which have failed to show any relationship (Koohmaraie et al., 1991; Whipple, Koohmaraie, Dikeman, Crouse, Klemm, & Hunt, 1990). The data presented by Alarcon-Rojo and Dransfield (1995) suggested that cathepsin D and cathepsins B + L may contribute to tenderisation late in the ageing period. At 6 days post-mortem, muscle strips soaked at 10°C from day 1 in a number of cathepsin inhibitors, were tougher than control samples. However enzymes such as cathepsin D have a reduced activity at chiller temperatures (Zeece, Katoh, Robson, & Parrish, 1986). Further to this, when Uytterhaegen et al. (1994) injected muscle with either pepstatin (60 μM) or a peptidyl-diazomethane (0.2 mM) at 1 day post-mortem these inhibitors of cathepsins D and B + L, respectively, did not inhibit ageing. The results of these workers clearly showed that catheptic enzymes could not be implicated in protein degradation in post-mortem muscle when held at chiller temperatures at least up to 8 days post-mortem. These results have been validated by those presented here.

5. Conclusions

It has been shown using an in situ approach that the calpains have a pivotal role in the proteolysis and tenderisation of meat post-mortem and that the cathepsins (B and L) are unlikely to contribute to these processes at least in the early post-mortem period. Osmolality per se did not explain significant variation in tenderness over and above the fixed effects used in the study.

It was apparent that rigor development momentarily overrode the beneficial effects of proteolysis as measured by shear force, and that subsequently the cumulative effect of proteolysis reversed the rise in toughness and significant tenderisation occurred. In the next paper in the series, further results will be presented and the impact of these on current theories of tenderisation discussed.

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References

Aalhus, J. L., Dugan, M. E. R., & Best, D. R. (1996). Calcium chloride — accelerates tenderisation or prevents toughening? *Meat Focus International*, *5*, 441–443

Alarcon-Rojo, A. D., & Dransfield, E. (1995). Alteration of post-mortem ageing in beef by the addition of enzyme inhibitors and activators. *Meat Science, 41*, 163–178.

Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M., & Hanada, K. (1982). L-*trans*-Epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochemistry Journal, 201*, 189–198.

Blackburn, S. (1968). *Amino acid determination methods and techniques*. London/New York: Edward Arnold/Marcel Dekker.

Chrystall, B. B., Devine, D. E., Ellery, S., & Wade, L. (1984). Low voltage electrical stimulation of lamb: its effect on muscle pH and tenderness. *New Zealand Journal of Agricultural Research, 27*, 513–523.

Dransfield, E. (1992). Modelling post-mortem tenderisation—III: role of calpain 1 in conditioning. *Meat Science, 31*, 85–94.

Dransfield, E. (1999). Meat tenderness — the μ-calpain hypothesis. In *Proceedings 45th International Congress of Meat Science and Technology* (pp. 220–228), Yokohama, Japan.

Dransfield, E., Etherington, D. J., & Taylor, M. A. J. (1992). Modelling post-mortem tenderisation: 11. Enzyme changes during storage of electrically stimulated and non-stimulated beef. *Meat Science, 31*, 75–84.

Etherington, D. J., Taylor, A. J., Wakefield, D. K., Cousins, A., & Dransfield, E. (1990). Proteinase (cathepsin B, D, L and calpains) levels and conditioning rates in normal, electrically stimulated and high-ultimate-pH chicken meat. *Meat Science, 28*, 99–109.

Feidt, C., Petit, A., Bruas-Reignier, F., & Brun-Bellut, J. (1996). Release of free amino-acids during ageing in bovine meat. *Meat Science, 44*, 19–25.

Fujishima, A., Imai, Y., Nomura, T., Fujisawa, Y., Yamamoto, Y., & Sugawara, T. (1997). The crystal structure of human cathepsin L complexed with E-64. *FEBS Letters, 40*, 747–750.

Hopkins, D. L., & Ferrier, G. R. (2000). The tenderness of lamb meat after low voltage stimulation under commercial conditions. *Asian–Australasian Journal of Animal Science, 13*(Suppl. July B), 356–357.

Hopkins, D. L., Littlefield, P. J., & Thompson, J. M. (2000a). A research note on factors affecting the determination of myofibrillar fragmentation. *Meat Science, 56*, 19–22.

Hopkins, D. L., Littlefield, P. J., & Thompson, J. M. (2000). Inhibition of protease activity 1. The effect on tenderness and myofibrillar fragmentation. In *Proceedings 46th International Congress of Meat Science and Technology* (pp. 452–453), Buenos Aires, Argentina.

Hopkins, D. L., & Thompson, J. M. (2001). The relationship between tenderness, proteolysis, muscle contraction and dissociation of actomyosin. *Meat Science, 57*, 1–12.

Kirschke, H., & Shaw, E. (1981). Rapid inactivation of cathepsin L by Z-Phe-PheChn$_2$ and Z-Phe-AlaChn$_2$. *Biochemical and Biophysical Research Communications, 101*, 454–458.

Koohmaraie, M., Whipple, G., Kretchmar, D. H., Crouse, J. D., & Mersmann, H. J. (1991). Postmortem proteolysis in longissimus muscle from beef, lamb and pork carcasses. *Journal Animal Science, 69*, 617–624.

McDonagh, M. B. (1998). *Calpain activity affects muscle protein turnover and meat tenderisation*. PhD thesis, University of New England.

Mobark, M., Connell, S., Reville, W., & Zeece, M. (1999). Localisation of lysosomal cathepsin B during the post-mortem storage of bovine muscle at 4°C and 25°C using enzyme fluorescence histochemistry. In *Proceedings 45th International Congress of Meat Science and Technology* (pp. 284–285), Yokohama, Japan.

O'Halloran, G. R., Troy, D. J., Buckley, D. J., & Reville, W. J. (1997). The role of endogenous proteases in the tenderisation of fast glycolysing muscle. *Meat Science, 47*, 187–210.

Oddy, V. H. (1974). A semiautomated method for the determination of plasma alpha amino nitrogen. *Clinica Chimica Acta, 51*, 151–156.

Ouali, A. (1992). Proteolytic and physicochemical mechanisms involved in meat texture development. *Biochimie, 74*, 251–265.

Parkes, C., Kembhavi, A. A., & Barrett, A. J. (1985). Calpain inhibition by peptide epoxides. *Biochemical Journal, 230*, 509–516.

Rich, D. A. (1986). Inhibitors of cysteine proteinases. In A. J. Barrett & G. Salvesen, *Research monographs in cell and tissue physiology — Proteinase inhibitors* (pp. 153–178). Oxford: Elsevier Science Publishers.

SAS (1997). *Applied statistics and the SAS programming language*. Cary, NC: SAS Institute, Inc.

Simmons, N. J., Singh, K., Dobbie, P.M., & Devine, C.E. (1996). The effect of pre-rigor holding temperature on calpain and calpastatin activity and meat tenderness. In *Proceedings 42nd International Congress of Meat Science and Technology* (pp. 414–415), Lillehammer, Norway.

Sugita, H., Ishiura, S., Suzuki, K., & Imahori, K. (1980). Inhibition of epoxide derivates on chicken calcium-activated neutral protease (CANP) *in vitro* and *in vivo*. *Journal of Biochemistry, 87*, 339–341.

Toldrá, F., Flores, M., & Aristory, M-C. (1995). Enzyme generation of free amino acids and its nutritional significance in processed pork meats. In G. Charalambous, *Developments in food science, food flavours 37B: Generation, analysis and process influence* (pp. 1303–1322). Amsterdam: Elsevier Science.

Uytterhaegen, L., Claeys, E., & Demeyer, D. (1994). Effects of exogenous protease effectors on beef tenderness development and myofibrillar degradation and solubility. *Journal of Animal Science, 72*, 1209–1223.

Varughese, K. I., Ahmed, F. R., Carey, P. R., Hasnain, S., Huber, C. P., & Storer, A. C. (1989). Crystal structure of a papain–E-64 complex. *Biochemistry, 28*, 1330–1332.

Whipple, G., Koohmaraie, M., Dikeman, M. E., Crouse, J. D., Hunt, M. C., & Klemm, R. D. (1990). Evaluation of attributes that affect longissimus muscle tenderness in *Bos taurus* and *Bos indicus* cattle. *Journal of Animal Science, 68*, 2716–2728.

Zeece, M. G., Katoh, K., Robson, R. M., & Parrish, F. C. Jr. (1986). Effect of cathepsin D on bovine myofibrils under different conditions of pH and temperature. *Journal of Food Science, 51*, 769–773.

Zeece, M. G., Woods, T. L., Keen, M. A., & Reville, W. J. (1992). Role of proteinases and inhibitors in postmortem muscle protein degradation. In *Proceedings 45th Annual Reciprocal Meat Conference* (pp. 51–61), Fort Collins, USA.