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The relationship between tenderness, proteolysis, muscle contraction and dissociation of actomyosin

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Abstract

The right side *m. longmissius thoracis et lumborum* (LTL) of 18 10-month-old male lambs was injected with the protease inhibitor E-64, and the left side was injected with isotonic saline within 30 min of stunning. Carcasses were hung during chilling by one of three methods; Achilles tendon; pubic symphysis or pubic symphysis with 2-kg weights attached to each hindleg. The LTL section was divided into three portions and aged for either 1, 3 or 7 days. The tenderness, myofibrillar fragmentation and dissociation of extracted actomyosin by pyrophosphate were determined for each portion of LTL. The protease inhibitor increased shear force values by 57%, with a decrease in shear force in the cranial-caudal direction along the LTL. Muscle from Achilles hung carcasses was the toughest. For sarcomere length hanging method had the greatest effect, with muscle from tenderstretched/weighted carcasses having the longest sarcomeres. Injection of an inhibitor caused a significant reduction in myofibrillar fragmentation ($P < 0.001$) and hanging method and ageing period also significantly affected this characteristic ($P < 0.05$). Osmolality of samples aged for 1 day was unaffected by any main effect. The amount of actomyosin (mg/g of muscle sample) extracted from muscles injected with the inhibitor was significantly less ($P < 0.001$) and there was also a significant effect ($P < 0.05$) of portion on this variable. The relationship between pyrophosphate and the percentage of myosin dissociated from the actomyosin complex was modelled using an exponential function; $Y = A - B \exp^{-kx}$. Analysis of A, B and k for the 69 samples fitted with an exponential function showed that there was no significant effect ($P > 0.05$) on A and k of any main effect or their interactions. There was, however, a significant effect ($P < 0.05$) of portion on B and also a significant interaction between injection and hanging method ($P < 0.05$). It was found that B did not explain any additional variance in shear force or myofibrillar fragmentation over the main effects and interactions previously found significant. The inhibitor E-64 was effective at preventing tenderisation indicating the role of cysteine proteases in proteolytic degradation. There was no apparent effect of dissociation on tenderness as measured in this experiment and therefore a causal relationship cannot be identified.

Keywords: Inhibitor; Proteases; Actomyosin; Tenderness; Lamb

1. Introduction

Specific muscle proteins are degraded during post-mortem ageing of muscle under chiller conditions (Bandman, Hwan, Suh-Foh & Zdanis, 1988; Wolfe & Samejima, 1976) and there is evidence that the cysteine proteases, in particular the calpains, are largely responsible for this degradation (Dransfield, 1993; Koohmaraie, 1996; Uytterhaegan, Claeys & Demeyer, 1994). The extent to which post-mortem changes in tenderness are due to degradation is widely debated, as the largest improvement in tenderness subsequent to rigor is observed within 24–48 h (Wheeler & Koohmaraie, 1994), yet myofibrillar protein degradation is not considered extensive during this period (Taylor, Geesink, Thompson, Koohmaraie & Goll, 1995). Indeed, Davey and Gilbert (1966) found that only 2.3% of meat proteins were degraded after 30 days of ageing at 2°C. Despite these findings it is plausible that degradation of strategic proteins may have a significant effect on meat structure and tenderness (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996).

It has been proposed that weakening of the bonds between actin and myosin may also contribute to the increase in tenderness observed in the immediate post-rigor period (Taylor et al., 1995). Supporting evidence that this interaction may impact on tenderness apart from its effect on contraction and fibre density was

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provided by Stein, Schwarz, Chock and Einsberg (1979) who proposed what is called the 'modified-refractory-state model' of muscle contraction in which there are both weak and strong cross-bridge states between actin and myosin. In a review of actin and actomyosin by dos Remedios and Moens (1995), it was stated "we must consider at least two states of actin-myosin binding, a high affinity and a low affinity state" and suggested that there are probably more than two. If this is so, then actomyosin has the potential to exist in different binding states at rigor and this may affect the ageing of meat and the absolute level of tenderness achieved. It is also claimed that the interaction between actin and myosin can be altered by the translocation of paratropomyosin under the influence of post-rigor rises in Ca^{2+} concentration (Takahashi, 1996).

One of the observations used to support the Ca^{2+} theory of tenderisation was the increase in sarcomere length post-rigor reported in some experiments (Stromer, Goll & Roth, 1967; Takahashi, Hattori & Kuroyanagi, 1995). This same observation was used by (Goll, Geesink, Taylor & Thompson, 1995) to suggest that change in the properties of actomyosin post-rigor point to other factors, which may affect tenderisation, although the calpains were recognised as having a major role in tenderisation. The increased ease with which actomyosin from aged rabbit muscle was dissociated by ATP compared to that from rigor muscle (Fujimaki, Arakawa, Okitani & Takagi, 1965) is one of the findings used to support the view that the interaction of actin and myosin impacts on tenderness. However, Wolfe and Samejima (1976) found no such change in either rabbit or chicken muscle and the data of Herring, Cassens, Fukazawa and Briskey (1969) for beef muscle was equivocal. In none of these studies was the possible effect of proteolysis on the interaction of actin and myosin, through degradation of troponin or other proteins accounted for. Troponin-T has been clearly shown to undergo degradation to produce a 30 Kda subunit (Ho, Stromer & Robson, 1994). Nebulin is also degraded (Anderson & Parrish, 1989; Watanabe & Devine, 1996) and Root and Wang (1994) have proposed that this protein was involved in the regulation of the interaction between actin and myosin.

Previous studies of the interaction of actin and myosin have not attempted to model the dissociation of actomyosin and relate this to measures of tenderness. This paper details a study of the interaction between actin and myosin in ovine muscle during the post-rigor period. To provide variation in actin/myosin overlap, muscles in different states of contraction at rigor were used and proteolysis was manipulated by injection of a cysteine protease inhibitor into the pre-rigor muscle. A preliminary report of some aspects of this study has been presented Hopkins, Littlefield and Thompson (1999).

2. Materials and methods

2.1. Animals and slaughter procedures

Eighteen second cross (Poll Dorset×Border Leicester×Merino) male lambs 10 months of age were used for the experiment. The lambs were slaughtered in three groups of six over a 6-week period. Prior to slaughter each group of six lambs were housed on a slatted floor and fed a wheat/lupin mix with pasture hay for 2 weeks before slaughter. At slaughter all 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. Standard dressing procedures were followed.

2.2. Injection procedure, hanging method and chilling

The protease enzyme inhibitor E-64 [*trans*-Epoxsuccinyl-L-Leucylamindo-(4-Guandino)] Butane (ICN Biomedicals Inc., OH, USA and Sigma-Aldrich Corporation, MO, USA) was injected into the *m. longmissius thoracis et lumborum* (LTL) on the right side of the carcass within 30 min of stunning, between the 12th/13th rib and the chump. To ensure an even distribution of inhibitor within the muscle, 50 ml of inhibitor was dispensed using a bank of 10 18-gauge needles, a total of 33 times. Each injection site received 0.15 ml with the delivery controlled using a multiple syringe pump, which delivered 18 mg of inhibitor per loin (1.0 mM) made up in isotonic saline (0.15 M NaCl). The left LTL (control) was injected with 50 ml of isotonic saline (0.15 M NaCl). Injection always commenced at the 12th/13th rib and progressed in a caudal direction. Preliminary study using a dye demonstrated that the injection method effectively dispersed the solution throughout the muscle. After injection carcasses were hung by either the, Achilles tendon; pubic symphysis, or pubic symphysis with 2-kg weights attached to each hindleg through the Achilles tendon to maximize stretch of the LTL.

Allocation of carcasses to the hanging treatment was randomized with two carcasses hung by each treatment on each slaughter day. Temperature decline was determined using Cox recorders (Belmont, NC, USA). Probes were inserted into the *m. semimembranosus* [(SM) to the depth of the femur] and the center of the LTL. Cold carcass weight was recorded and fat depth over the 12th rib measured.

2.3. Sample collection and preparation

The carcasses were chilled at 5°C for between 16 and 21 h and then the injected portion of the LTL removed from both sides of the carcass. A portion of the uninjected LTL between the 10th and 12th rib was also removed and frozen on day 1. Injected sections of the LTL from both sides of the carcass were divided into three

portions (cranial, medial and caudal) and randomly assigned to ageing periods of 1, 3 or 7 days. Portions to be aged were vacuum packed and held at 2.3°C.

After the appropriate ageing period, a 5-g muscle sample was taken for actomyosin extraction. For the remainder of the portion, the subcutaneous fat and epimysium were removed and a block trimmed to 65 g prior to storage at −20°C. Samples were also kept frozen for SDS electrophoresis, measurement of osmolality, myofibrillar fragmentation assessment (MFI) and measurement of sarcomere length. 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 allow subsequent separation of fibres along the direction of the myofibrils.

2.4. Measurement of shear force, sarcomere length, pH and osmolality

The 65-g LTL samples were cooked from frozen for 35 min in plastic bags at 70°C in an 80-l waterbath. From each block, six sub-samples with a cross-sectional area of 1 cm^2 were cut parallel to the muscle fibres and peak force measured using a Lloyd (Model LRX, Lloyd Instruments, Hampshire, UK) with a Warner–Bratzler shear blade fitted (Bouton, Harris & Shorthose, 1971). After cooking the samples were cooled in cold running water for 30 min, dried with paper towel and weighed. Cooking loss was calculated by dividing the cooked weight by the frozen weight, subtracting this value from 100 and expressing values as percentages. Sarcomere length was measured on sections of frozen LTL using laser light diffraction as reported by (Bouton, Fisher, Harris & Baxter, 1973).

The retained portion of the uninjected LTL between the 10th and 12th ribs was thawed and used for measurement of ultimate pH using an Orion meter (Model 250 A, Orion Research International, MA, USA) with a Ionode IJ 42 electrode. Muscle juice for determination of osmolality was obtained by centrifuging 2.5 g of finely chopped LTL at 100,000 *g* (Model L8-M, Beckman Instruments, CA, USA) for 20 min after (Ouali, Vigon & Bonnet, 1991) avoiding fat and conective tissue. The fresh juice was used for osmolality determination by measurement of osmotic pressure using an osometer (5500 Osometer, Wescor, UT, USA). The osometer was calibrated with standards having an osmolality of 290 and 1000 mmol/kg, in that order and then calibration at the low end of the curve checked with the 290 mmol/kg standard. Samples of LTL from each portion held for 1 day post-mortem were tested in triplicate.

2.5. Myofibrillar fragmentation index (MFI)

A thin slice of frozen muscle from each portion of the LTL was used for determination of MFIs based on a modification of the procedure of Culler, Parrish, Smith and Cross (1978) and described by Hopkins et al. (2000a).

2.6. Protein extraction

Actomyosin was extracted using a modified method to that described by Briskey and Fukazawa (1971) which is based on salt solublization of the proteins. Briefly, the main technique modifications were use of a protease inhibitor cocktail during the initial homogenization of the muscle, re-homogenization of the insoluble pellet, an extraction solution with a pH of 7.4 and faster centrifugation speeds throughout the procedure.

A 5-g sample of LTL was taken from each portion avoiding visible fat and connective tissue and homogenized in 15 ml of 0.6 M KCl solution containing 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3, 1 mM EDTA and a protease inhibitor. The effective concentrations of the various components in the protease inhibitor were; AEBSF (139 μM), Bestatin (5.6 μM), E64 (1.9 μM), Leupeptin (3.0 μM), Aprotinin (0.1 μM) and Pepstatin (2.1 μM) (Sigma-Aldrich Corporation, MO, USA). The pH of the solution was adjusted to 7.4 with HCl. Samples were homogenized for three bursts of 15 s with a 30-s break on ice between using an Ultra-Turrax homogeniser (Dottingen, Germany) operating at 25,000 rpm. The homogensier shaft was washed with 30 ml of extraction solution and the suspension gently agitated for 24 h at 2°C on a roller mixer. After extraction the suspension was centrifuged at 1000 *g* for 15 min using a Sorvall® Superspeed centrifuge (Model RC2-B, DuPont Instruments). The pellet was homogenized in 10 ml of cold 0.6 M KCl solution for 15 s and the suspension centrifuged at 1000 *g* for 15 min. The supernatants were combined and diluted with cold deionized water to a final concentration of 0.16 M KCl and held chilled for 30 min. The samples were then centrifuged for 20 min at 5000 *g* and the supernatant discarded. The pellet was dissolved in 25 ml of cold 3 M KCl with 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3 and 1 mM EDTA (pH adjusted to 7.4 with HCl). The suspension was gently agitated for 30 min at 2°C on the roller mixer then diluted with cold deionized water to a concentration of 0.6 M KCl. After centrifugation for 15 min at 1000 *g* the retained supernatant was diluted to 0.16 M KCl with cold deionized water and the solution mixed and divided into two equal volumes which were centrifuged for 15 min at 1000 *g*. The supernatant was discarded and the precipitate dissolved in 15 ml of cold 2.4 M KCl with 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3, 1 mM EDTA (pH adjusted to 7.4 with HCl) then diluted with cold deionized water to 0.15 M KCl. After centrifugation for 15 min at 1000 *g* the supernatant was discarded and the step repeated. For the final step the precipitate was

dissolved in 10 ml of cold 3 M KCl solution then diluted with cold deionized water to drop the concentration to 0.6 M KCl. This sample was centrifuged for 60 min at 25,400 *g*. Two 20-ml volumes of the protein solution were stored with equivalent amounts of glycerol at −20°C and approximately 5 ml of the protein solution was frozen at −20°C for subsequent SDS electrophoresis and protein determination.

2.7. Dissociation of actomyosin

The glycerol was removed from the stored actomyosin suspensions for 93 of the 108 samples by precipitating the actomyosin with an equal volume of cold deionized water under centrifugation (5000 *g* for 20 min). The 15 samples which were not used, were either frozen during storage, or had very low protein concentrations. The protein concentration of actomyosin suspensions was determined in triplicate at 562 nm, prior to dissociation using the BCA Protein assay kit (Pierce Chemical Company, IL, USA). The protein concentration was adjusted to 0.3 mg/ml using a buffer (0.6 M KCl, 4 mM $MgCl_2$, 0.02 M Tris, pH adjusted to 6.8 with acetic acid). Aliquots of the protein suspensions were mixed with either buffer or with $Na_2H_2P_2O_7$ (disodium pyrophosphate) at effective concentrations of either 0.025, 0.05 or 0.5 mM. The effective protein concentration after mixing was reduced to 0.26 mg/ml. The solutions were then subjected to ultracentrifugation for 3 h at 100,000 *g* at 5°C after Fujimaki et al. (1965). Aliquots of protein from the supernatant were taken in triplicate and the protein concentration measured. Absorption was measured at 570 nm using a microplate reader (Titertek Multiskan® Plus, Lab Systems OY, Helsinki, Finland) in accordance with the specified protocol. A standard curve was based on BSA at a range of concentrations.

2.8. Electrophoresis

Fifteen actomyosin samples representing the concentration range of those extracted were dialyzed against a solution containing 0.1% (w/v) SDS, using dialysis tubing with a molecular weight cut-off of 12–14,000 kDa. Dialysis was conducted overnight under chiller conditions with agitation provided by a magnetic stirrer. This approach was necessary to avoid precipitation of the SDS in the buffer solution with the K^+ ions in the extraction solution as reported by Chalmers, Careche and Mackie (1992). The protein concentration in the dialyzed solutions was increased by use of centrifugal concentrators with a molecular weight cut off of 10 Kda (Vivaspin 15, Vivascience Ltd, Lincoln, UK). Concentrators were centrifuged at 2000 *g* for up to 45 min in a Beckman centrifuge (Model CPR, Beckman Instruments, CA, USA) at 5°C. The filtrate was decanted and the concentrated solution retained. Aliquots (50 μl) were taken and the protein concentration determined in triplicate using BSA for the standard curve and the BCA method. The concentration of protein was adjusted to 0.4 mg/ml for each sample, which was the lowest level, measured.

A 50-μl aliquot of each sample was then taken and diluted with 50 μl of an SDS buffer [10 mm Tris/HCl, 1 mM EDTA, 2.5% SDS (w/v), 5.0% β-mercaptoethanol and 0.01% bromophenol blue (both v/v), adjusted to pH 8.0] made up as double strength. An internal BSA standard was used in the solutions with an effective concentration of 0.1 mg/ml. The solutions were then heated for 5 min at 90°C in a Dri-Bath (Type 17600, Thermolyne Corporation, IA, USA) and centrifuged at 800 *g* for 2 min. A 2-μl aliquot of each sample was loaded onto homogeneous mini gels containing 12.5% polyacrylamide (PhastSystem, Amersham Pharmacia Biotech, Uppsala, Sweden) at 250 V at 15°C. These gels have a 13 mm stacking zone and a 32 mm separation zone with 3 and 2% cross-linking, respectively. The gels are 0.45 mm thick and the buffer strips contain 0.2 M tricine, 0.2 M Tris, 0.55% SDS with a pH of 8.1. Proteins were stained by developing gels at 50°C in a 0.1% Coomassie Blue R_{250}, 20% acetic acid solution using the PhastSystem™. Gels were destained in a solution containing 30% methanol and 10% acetic acid (v/v) and preserved in a solution containing 5% glycerol and 10% acetic acid (v/v) both made up in deionized water.

Markers (Pharmacia LKB, Uppsala, Sweden) of known molecular weight were run with gels to aid identification of proteins. These were phosphorylase (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400), all in sucrose.

Images of the gels were captured using an image processing and analysis system (Leica Q500MC, Leica Cambridge Ltd, UK) linked to a CCD camera. Actin, myosin and BSA bands were selected and using a binary image the area of each band was determined. Image sharpness and contrast were adjusted for presentation purposes using Paint Shop Pro V 3.0 (JASC, Inc. MN, USA).

2.9. Statistical analysis

Shear force measurements, sarcomere length, MFI, osmolality and the amount of extracted actomyosin from the LTL were analysed using a mixed model with main effects for hanging method (achilles tendon, tenderstretched and tenderstretched weighted), age of sample (1, 3 and 7 days post-mortem), portion (cranial, medial and caudal) and injection (inhibitor and control), with animal nested within hanging method as a random term. First order interactions were tested. In the case of osmolality data, age of sample was not

tested. Predicted means were compared using the PDIFF statement, which enables multiple comparisons.

The relationship between pyrophosphate concentration and the percentage of myosin dissociated from the actomyosin complex was modelled using an exponential function;

$$Y = A - B\exp^{-kx}$$

where Y = percentage of protein dissociated from the complex and x = the concentration of pyrophosphate, A is the asymptote and B is the difference between the value of Y at the highest concentration of pyrophosphate and the value of Y when $x = 0$. k = rate of dissociation.

Of the 93 samples subjected to the dissociation tests, the model only converged for 69 using a non-linear procedure (S-Plus, 1997). The parameters (A, B and k) from the non-linear models were then analysed using a mixed model SAS (1997) which contained the main effects and their interactions. The data for 93 samples were also subjected to a repeated measures analysis (Gilmour, 1996), where the dependent variable comprised percentage protein dissociated at 0, 0.025, 0.05 and 0.5 mM of pryophosphate in a model which contained all main effects and their first order interactions.

Equations were developed using a non-linear procedure SAS (1997) to describe the relationship between muscle temperature and time, according to muscle, using the function;

$$\text{Temperature} = T_f + (T_i - T_f)\exp^{-kt}$$

where T_i = the temperature at time zero and T_f = the final temperature, t = the time in hours and k = rate constant of temperature decay.

Estimates of T_i, T_f and k were derived for each carcass and both muscles. These parameters were then tested against the factor, hanging method using the mixed model procedure with a random term, animal nested within treatment, included. The temperature at 8 h postmortem was also predicted for each carcass and muscle and then tested against the factor hanging method as for the parameters.

3. Results

3.1. Carcass characteristics and chilling rate

The mean (± S.D.) carcass weight and fat depth as measured at the 12th rib was 29.6 ± 3.1 kg and 3.5 ± 1.5 mm, respectively. The mean (± S.D.) ultimate pH was 5.6 ± 0.06.

There was no significant difference ($P > 0.05$) between hanging methods for the rate constants (k) of decline in temperature in either muscle. Predicted temperature at 8 h was not significantly different ($P > 0.05$) between hanging methods for either muscle. The correlation between muscle temperatures in the two muscles at 8 h was 0.68 ($P < 0.05$).

3.2. Objective meat quality measurements

There was no significant effect ($P > 0.05$) of ageing on shear force values, but injection, muscle portion and hanging method all had a significant effect ($P < 0.001$) in that order of importance. Of the first order interactions, injection × hanging method was significant ($P < 0.001$) as was injection × portion and portion × hanging method ($P < 0.05$). Predicted shear force means for the main effects and significant interactions are shown in Table 1. For sarcomere length, injection and muscle portion were significant ($P < 0.05$) as was hanging method ($P < 0.001$; Table 2). There was a significant interaction between hanging method and ageing period ($P < 0.05$).

Injection had a significant effect on myofibrillar fragmentation ($P < 0.001$) as did hanging method and ageing period ($P < 0.05$). Portion had no significant effect on myofibrillar fragmentation, nor did any of the first order interactions. The only significant effect ($P < 0.05$) on cooking loss was the method of hanging (Table 3).

The correlation between myofibrillar fragmentation index and shear force values was $r = -0.41$ ($P < 0.001$). There was no significant effect ($P > 0.05$) of injection, hanging method, portion or the first order interactions on osmolality for samples aged for 1 day.

3.3. Actomyosin extraction and dissociation

The amount of actomyosin (mg/gram of muscle sample) extracted from muscles injected with the inhibitor was significantly less ($P < 0.001$) and was affected ($P < 0.001$) by portion (Table 4). There were no other significant effects on the amount of actomyosin extracted.

A plot of the mean data for the proportion of dissociated protein from the actomyosin complex as a function of pyrophosphate concentration is shown in Fig. 1.

Analysis of A, B and k for the 69 samples fitted with an exponential function showed that there was no significant effect ($P > 0.05$) on A and k of any main effect or their interactions. There was however a significant effect ($P < 0.05$) of portion on B (Table 5) and also a significant interaction between injection and hanging method ($P < 0.05$). The same result was found when the difference in the proportion of dissociated protein between pyrophosphate at 0.5 mM and the blank was used. This indicates the estimates of B provided a meaningful explanation of this aspect of dissociation.

From the repeated measures analysis, there was also a significant effect of portion on the amount of dissociated

Table 1
Predicted means (S.E.) of Warner–Bratzler (WB) shear values adjusted for main effects and significant interactions ($n = 108$)[a,b]

Terms	df[c]	*F*-ratio	Level	WB (kg)			Average S.E.
Injection	1/77	311.5***	Inhibitor	6.6 x			
			Control	4.2 y			0.18
Portion	2/77	35.3***	Cranial	6.0 x			
			Medial	5.4 y			
			Caudal	4.6 z			0.19
Hanging method	2/15	17.4***	Achilles tendon	6.6 x			
			Tenderstretched	5.3 y			
			Tenderstretched/ weighted	4.2 z			0.28
Ageing	2/77	0.44					
			Hanging method				
			Injection	Achilles tendon	Tenderstretched	Tenderstretched/ weighted	
Injection×hanging method	2/77	12.9***	Control	5.1 ay	3.9 bcy	3.5 cy	
			Inhibitor	8.1 ax	6.7 bx	4.9 cx	0.31
			Portion				
Portion×hanging method	4/77	3.4*	Cranial	7.7 ax	5.8 bx	4.6 cx	
			Medial	6.4 ay	5.3 bx	4.3 cx	
			Caudal	5.5 az	4.7 ay	3.7 cy	0.33
			Injection				
				Inhibitor	Control		
Injection×portion	2/77	6.6*	Cranial	6.9 ax	5.2 bx		
			Medial	6.6 axy	4.1 by		
			Caudal	6.1 ay	3.2 bz		0.22

[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] df, degrees of freedom (numerator/denominator).
***$P < 0.001$; *$P < 0.05$.

protein. Predicted means for the percentage of dissociated protein at each concentration of pyrophosphate were determined for each portion and the plot of these is shown in Fig. 2. This showed a greater change in the amount of protein measured in the supernatant between solutions with no pyrophosphate and those with 0.05 mM pyrophosphate, for samples from the caudal portion. It also showed a slight decline in the amount of dissociated protein for this portion as the pyrophosphate concentration increased to 0.5 mM, whereas for the other two portions this did not occur. This effect was partly responsible for the fact that not all samples could be fitted by an exponential function.

Given the effect of LTL portion on shear force values and on the parameter *B*, an analysis was performed to examine whether *B* could explain any of the variance in shear force for the reduced sample size of 69. This showed that *B* did not explain any additional variance in shear force over the main effects and interactions previously found significant. There was in fact no correlation between *B* and shear force ($r = -0.003$). This approach was extended to MFI data and this also showed no significant relationship between this variable and *B*.

Using electrophoresis samples representing the range of actomyosin extracted per g of muscle were examined. Of the 15 samples subjected to dialysis, seven were selected randomly to cover the range in the amount of actomyosin extracted (w\w) and their protein bands are shown in Fig. 3. This indicated at least visually that the ratio of actin to myosin was similar between samples, apart from the sample represented in lane f. The relative amounts were semiquantified by the calculation of the area of each actin, myosin and BSA band.

Given that the concentration of BSA loaded onto each lane was theoretically the same, the area of the smallest band (lane b) was used as a scaling factor for the other BSA bands and this used to adjust the area of each band for actin and myosin. The ratio of the area of myosin to actin was then calculated and this is indicated in Fig. 3. This confirmed that the sample in lane f (muscle injected with saline, aged 1 day, tenderstretched, cranial portion), had a low ratio of extracted myosin to actin and vice versa for the sample in lane b

Table 2
Predicted means (S.E.) for sarcomere length adjusted for main effects and significant interactions[a,b]

Terms	df[c]	F-ratio	Levels	Sarcomere length (μm)	Average S.E.
Hanging method	2/15	19***	Achilles tendon	1.87 x	
			Tenderstretched	1.96 x	
			Tenderstretched/ weighted	2.15 y	0.032
Injection	1/81	8.3*	Inhibitor	2.03 x	
			Control	1.96 y	0.022
Portion	2/81	6.5*	Cranial	1.93 x	
			Medial	2.02 y	
			Caudal	2.03 y	0.026
Ageing	2/81	2.1			

Hanging method

Terms	df	F-ratio	Ageing (days)	Achilles tendon	Tenderstretched	Tenderstretched/weighted	
Hanging method ×age	4/81	2.6*	1	1.84 ax	2.01 bcx	2.07 cx	
			3	1.87 ax	1.88 ay	2.17 bxy	
			7	1.89 ax	1.98 byx	2.20 cy	0.045

[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] df = degrees of freedom (numerator/denominator).
***$P < 0.001$; *$P < 0.05$.

(muscle injected with inhibitor, aged 7 days, Achilles hung, caudal portion) with the other samples being similar. The origin of the samples represented in each lane is shown in Fig. 3.

4. Discussion

4.1. Effects of protease inhibition

Shear force data in this study clearly showed that the cysteine inhibitor E-64 was effective at inhibiting the action of cysteine active proteases with a 57% increase in toughness over the three hanging treatments. The *F*-ratios indicated that injection was also the most important factor in accounting for the variance in shear force values. Uytterhaegan et al. (1994) found that a range of cysteine protease inhibitors including E-64 injected into muscle at 24 h post-mortem effectively maintained shear force values at those equivalent to day 1 samples in agreement with the response found by us. In contrast to our results, injection of inhibitors has not always been reported to prevent tenderisation (Aalhus, 1994; Aalhus, Dugan & Best, 1996), although these workers did acknowledge that problems with permeability and diffusion of the inhibitors might have influenced the results. This was supported by our results where we had verified dispersal of the injected solution throughout the muscle and also the results of Uytterhaegan et al. (1994), where the use of small cores would have ensured a good distribution of inhibitor.

Our data clearly showed that the solubility of actomyosin decreased in injected samples, and it could be postulated that this was due to a reduction in the

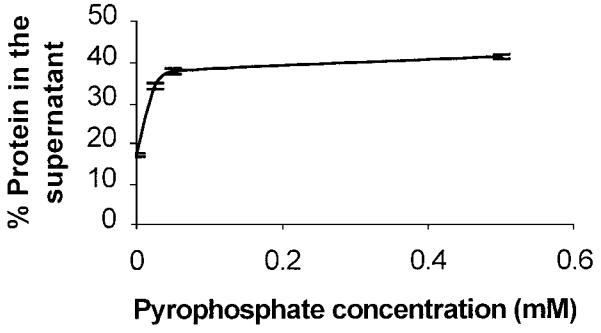

Fig. 1. A plot of the mean (± S.D.) percentage of dissociated protein versus pyrophosphate concentration (mM) for all samples tested for dissociation ($n = 93$).

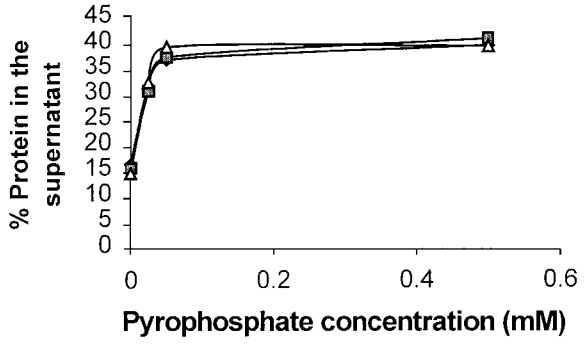

Fig. 2. A plot of the predicted percentage of dissociated protein versus pyrophosphate concentration (mM) according to portion (cranial = ◆, medial = ■, caudal = Δ).

fragility of major structural proteins that connect actomyosin to the Z-disk such as titin (Tanabe, Tatsumi & Takahashi, 1994) and a reduction in proteolysis. The lower MFI values for injected muscle supported this conclusion. We did, however, find an overall increase in MFI values due to ageing, indicative of proteolysis, but as for shear force it was injection that had the dominant influence on MFI values as indicated by the *F*-ratios. It has been established that changes in MFI values are indicative of degradation of proteins in the I-band of the sarcomere (Taylor et al., 1995) a region where nebulin and titin are known to be degraded (Boyer-Berri & Greaser, 1998; Furst, Osborn, Nave & Weber, 1988). The significant reduction in MFI values for injected muscle in our study was in line with a reduction in degradation in this region of the sarcomere. In the work of Uytterhaegan et al. (1994) limited degradation of proteins such as titin, nebulin and troponin-T in meat injected with E-64 occurred, as assessed by SDS electrophoresis. Under denaturing gel electrophoresis

Table 3
Predicted means (S.E.) for myofibrillar fragmentation index and cooking loss adjusted for significant main effects[a]

Terms		Myofibrillar fragmentation index (MFI)				Cooking loss (CL) (%)			Osmolality (Os)[b]		
	Levels	df[c]	*F*-ratio	MFI	Average S.E.	*F*-ratio	CL (%)	Average S.E.	*F*-ratio	Os (mmol/kg)	Average S.E.
Injection	Inhibitor	1/85	19.2***	86 x		3.0			1.6	465 x	
	Control			96 y	1.6					470 x	3.8
Hanging method	Achilles tendon	2/15	7.0*	85 x		5.8*	23.2 x		1.7	471 x	
	Tenderstretched			93 y			21.6 xy			473 x	
	Tenderstretched/ weighted			95 y	2.0		20 y	0.66		459 x	5.7
Ageing	1	2/85	5.4*	86 x		0.2					
	3			91 xy							
	7			96 y	2.0						
Portion		2/85	0.4			2.7			1.6	469 x	
										462 x	
										472 x	4.7

[a] Values followed by the same letter in a column within a main effect (x, y, z) are not significantly different ($P < 0.05$).
[b] No significant effect for osmolality.
[c] df, degrees of freedom (numerator/denominator).
***$P < 0.001$; *$P < 0.05$.

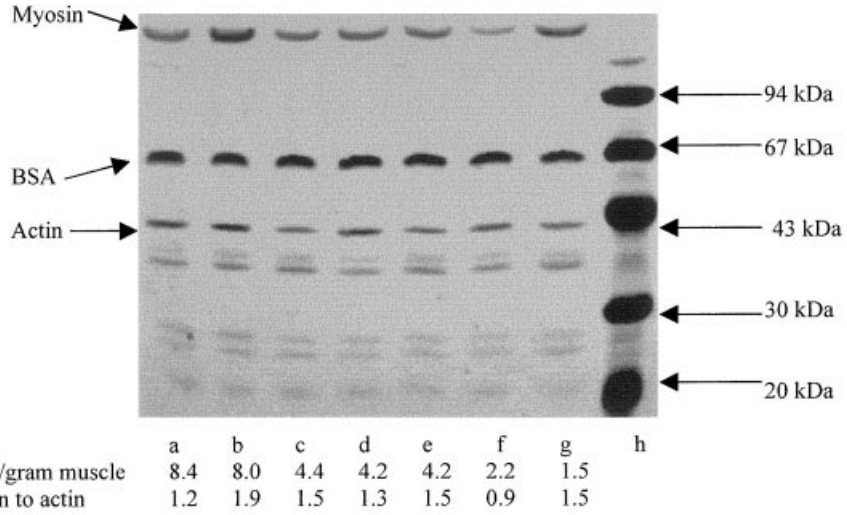

Fig. 3. Electrophoresis pattern for proteins extracted as actomyosin from muscle samples taken from carcasses hung by different methods and injected with either the protease inhibitor (E-64) or isotonic saline and aged for different periods. lane h, low molecular weight markers, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). lane a, muscle injected with saline aged for 3 days (Achilles hung, caudal portion); lane b, muscle injected with inhibitor, aged 7 days (same carcass as lane a, caudal portion); lane c, muscle injected with inhibitor, aged 3 days (tenderstretched, medial portion); lane d, muscle injected with saline, aged 3 days (Achilles hung, cranial portion); lane e, muscle injected with inhibitor, aged 1 day (tenderstretched, medial portion); lane f, muscle injected with saline, aged 1 day (tenderstretched, cranial portion); lane g, muscle injected with inhibitor, aged 7 days (tenderstretched, medial portion).

Table 4
Predicted means (S.E.) for the amount of actomyosin extracted per g of wet muscle (AM) when adjusted for significant main effects[a]

Terms	df[b]	*F*-ratio	Levels	AM (mg/g)	Average S.E.
Injection	1/85	27.1***	Inhibitor	3.6 x	
			Control	5.1 y	0.22
Portion	2/85	24.2***	Cranial	3.0 x	
			Medial	4.5 y	
			Caudal	5.5 z	0.26
Hanging method	2/15	0.4			
Ageing	2/85	0.5			

[a] Values followed by the same letter in a column within a main effect are not significantly different ($P < 0.05$).
[b] df, degrees of freedom (numerator/denominator).
***$P < 0.001$; *$P < 0.05$.

conditions we also observed the appearance of the 30 kDa fragment in some muscle samples injected with both inhibitor, or saline after ageing for three or more days (data not shown). As such it was apparent, that proteolysis did occur in control samples, even though there was no reduction in shear force values with ageing. Given the magnitude of the MFI values for day 1 samples and the overall mean shear force for control samples (4.2 kg) it seems evident that significant ageing had occurred prior to the first sampling. In fact it was evident from electrophoresis that some proteolysis even occurred in inhibited samples. To our knowledge, our data is the first to document the effect of a broad ranging cysteine protease inhibitor on MFI values for ovine muscle. Although we accept that an increase in ionic strength associated with the development of rigor may contribute to the loss of the integrity of myofibrils Ouali et al. (1991), there was no basis to suggest this in any way caused the effect ascribed to the inhibitor, as osmolality did not differ between treatments.

4.2. Impact of sarcomere length

The muscle stretching techniques employed in this study were effective in altering sarcomere length (1.87–2.15 μm for the normally hung and weighted tenderstretch treatments, respectively) and had the greatest effect on sarcomere length (evidenced by the largest *F*-ratio). Tenderstretching was used as a method of altering the 'state' of bonding between actin and myosin in order to study the changes in the actomyosin complex. Electron microscope images confirmed that the overlap between actin and myosin was reduced at rigor in stretched muscle from this experiment (Hopkins et al., 2000). As a consequence of the stretching methods used in this study there was a significant effect on shear force values. We are only aware of the one report by Eikelenboom, Barnier, Hoving-Bolink, Smulders and Culioli, (1998) where leg weighting of tenderstretched carcasses has been used to maximize stretch in vivo as we did. The response in terms of sarcomere length was similar.

In our study, the interaction between method of hanging and injection of an inhibitor showed that hanging the carcass from the pubic symphysis and weighting the leg reduced shear force in muscle treated with inhibitor by 3.2 kg compared with only 1.6 kg in the control muscle (Table 1). Related to this, the difference in shear force between inhibitor and control muscle was significantly reduced from 3 kg in Achilles hung carcasses to 1.4 kg in carcasses hung by the pubic symphysis with weighting of the leg. This showed that the increase in toughness of inhibited muscle was halved

Table 5
Predicted means (S.E.) for the parameter B (% dissociated protein), which is the difference between the value of Y at the highest concentration of pyrophosphate and the value of Y when x = 0 derived from the exponential equation $Y = A - B\exp^{-kx}$ and adjusted for significant main effects and interactions[a,b]

Terms	df[c]	*F*-ratio	Levels	*B*			Average S.E.
Portion	2/44	4.1*	Cranial	−21.6 x			
			Medial	−25.6 y			
			Caudal	−27.1 y			1.35
Injection	1/44	1.0					
Hanging method	2/15	0.8					
Ageing	2/44	0.5					
			Hanging method				
			Injection	*Achilles tendon*	*Tenderstretched*	*Tenderstretched/weighted*	
Hanging method × injection	2/44	3.6*	Inhibitor	−25.6 ax	−26.5 ax	−19.9 bx	
			Control	−25.4 ax	−24.3 ax	−27.0 ay	1.86

[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 effect or interaction (x, y, z) are not significantly different ($P < 0.05$).
[c] df, degrees of freedom (numerator/denominator).
*$P < 0.05$.

if the muscle was stretched using weights and as such indicates that fibre density has a large effect on shear force. Based on the muscle temperatures recorded in our study the chilling regime could be classified as medium to slow (Shaw, Eustace & Smith, 1995) and therefore not an explanation for the lack of reduction in shear force values for control samples after day 1. It is of interest that pre-rigor stretching significantly increased MFI values, indicating increased fragility of proteins such as titin as a result of this treatment.

4.3. Dissociation of actomyosin

For our data, there was no evidence that stretching or ageing had an affect on the dissociation of actomyosin, indicated by our modeling of the dissociation curve. This modeling was in contrast to previous studies where interpretation was based on qualitative examination of dissociation curves (e.g. Fujimaki et al., 1965). A constraint in our modeling was the number of different levels of pyrophosphate used for dissociation as this did affect the ability to fit all data points. However, scrutiny of the curves published by Wolfe and Samejima (1976) suggests that a number of these would also not have conformed to an exponential function. Our results are in contrast to those of Fujimaki et al., (1965), but supported by those of Wolfe and Samejima when they dissociated rabbit and chicken actomyosin with either ATP or pyrophosphate and also by Arakawa, Inagaki, Kitamura, Fujiki and Fujimaki (1976) who used myosin from rabbit muscle myofibrils.

In none of these studies was there any attempt to model the dissociation of actomyosin and relate this to measures of tenderness such as shear force or indicators of proteolysis such as myofibrillar fragmentation. For our data that did conform to the exponential pattern, the parameters were mostly unaffected by the treatments used in the experiment. Muscle portion and the interaction between injection and hanging method both had an effect on dissociation and shear force. The lack of change in shear force in control muscles hung by the Achilles tendon was unexpected and may have contributed to the lack of relationship between dissociation and tenderness. However, the MFI, data did indicate that significant degradation occurred over the 7 days of ageing and indeed after injection, but before first sampling. Our stretching methodology did reduce the overlap of actin and myosin and was an attempt to alter the 'state' of bonding between the two proteins at rigor, yet this did not impact on the degree of dissociation. It should be stressed however that given evidence from electrophoresis that the ratio of actin to myosin was not the same in every actomyosin sample, this might have altered the response of such samples to pyrophosphate. However, given that the ratio varied between samples treated in very different ways, this effect was not considered to have had a major bearing on the responses observed.

The use of the inhibitor meant that any effect of the degradation of troponin or nebulin on the interaction of actin and myosin was likely eliminated or significantly reduced. It is possible that in past studies degradation may have impacted on the results, given the proposal by Root and Wang (1994) that nebulin is involved in the regulation of the interaction of actin and myosin. It has also been shown that troponin-T degradation occurs as muscle ages (Ho et al., 1994), and Taylor et al. (1995) has suggested that this might also effect the interaction of actin and myosin and more generally myofibril integrity (Huff-Lonergan et al., 1996).

Certainly the inhibitor in our study significantly reduced tenderisation, but this had no effect on the degree of dissociation. Indeed, there was no evidence that the strength of actomyosin bonding had a major impact on post-rigor tenderness as measured by shear force, given the design of the experiment. However, portion effects may in part be explained by changes in the ease with which actomyosin could be dissociated, but this effect was not large enough to impact on shear force over and above the effects of proteolysis and fibre density.

5. Conclusions

The inhibitor E-64 was effective at preventing tenderisation indicating the role of cysteine proteases in proteolytic degradation. There was no apparent effect of dissociation on tenderness as measured in this experiment and therefore a causal relationship cannot be identified. Further study of the interaction between actin and myosin would be beneficial if a method could be developed to manipulate the 'state' of bonding of the complex at rigor, this being a significant biochemical challenge.

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