INHIBITION OF PROTEASE ACTIVITY 1. THE EFFECT ON TENDERNESS AND MYOFIBRILLAR FRAGMENTATION

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Background.

Specific muscle proteins are degraded during post-mortem ageing of muscle at chiller temperatures and there is evidence that the cysteine proteases, in particular the calpains, are responsible for myofibrillar protein degradation *(Uytterhaegen et al., 1994)*. There is debate, however about the role of the other major group of cysteine proteases, the cathepsins. *Ouali (1992)* reviewed mechanisms controlling post-mortem tenderisation and cited results from experiments where both rabbit and beef muscles were studied, which suggested that the cathepsins did contribute to degradation. However *Koohmaraie et al. (1991)* showed that although lamb and beef had similar cathepsin B and B + L activity measured at death, the rate of tenderisation in the different species was very different suggesting that the cathepsins made little contribution to degradation of myofibrillar proteins. By contrast, *O'Halloran et al. (1997)* suggested that the higher activity of 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.

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 *in vitro* conditions *Sugita et al. (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 E-64 significantly reduced tenderisation if effectively delivered into muscle *(Hopkins et al., 1999)*. Also using a thorough method of injecting inhibitors into muscle, *Uytterhaegan et al. (1994)* found that a range of inhibitors including E-64, injected at 24 hours post-mortem effectively stopped the ageing process. *Uytterhaegan 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. This provided strong evidence for the role of the calpains in meat tenderisation, although *Dransfield (1999)* suggested, that the role of the calpains and cathepsins in tenderisation had not been clarified by the use of inhibitors.

Objective.

This study 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.

Methods.

Animals and slaughter procedures: Twenty-four male lambs aged 8 months were slaughtered in 3 groups of 8, over a 3-week period under low stress conditions. Within 15 minutes of death E-64 (a broad-spectrum inhibitor for both calpain and cathepsin proteases) was injected into the m. *longmissius lumborum* (LL) on the right side of 12 carcasses (4 per slaughter) between the $12^{th}/13^{th}$ rib and the tuber coxae. To ensure an even distribution of the inhibitor (1.4 mM) within the muscle it was dispensed in a saline (0.25 M NaCl), 20% dimethyl sulfoxide solution using a multiple syringe pump with a bank of 20 needles. In another 12 carcasses (4 per slaughter) the protease inhibitor Z-Phe-Ala-CHN_2 (N-carbobenzoxy-phenylalanyl-alanyl-diazomethylketone) which is specific to cathespins B and L was injected using the same procedure at a concentration of 0.2 mM *(Uytterhaegan et al. 1994)*. The left side of all carcasses served as the control and was injected with an equal volume of the saline (0.25 M NaCl), 20% dimethyl sulfoxide solution. On each slaughter day every second carcass was subjected to low voltage stimulation immediately after dressing and before injection (45 V, for 40 secs, with 36 pulses per sec and a pulse width of 25 millisecs). Carcasses were then chilled at 6°C for 17-22 hours. The injected portion of the LL was removed from both sides, divided into 2 portions and randomised into 2 ageing treatments. One section was frozen (-20°C) at 24 hours post-mortem and the other vacuum packed and aged until 48 hours post-mortem at 2.3°C. After ageing each portion was trimmed of epimysium to a 65g block for shear testing and frozen at -20°C. At the same time a 5-gram sample was taken for determination of myofibrillar fragmentation and frozen at -20°C.

Meat quality measurements: The LL samples were cooked from frozen and peak force measured *(Hopkins et al., 1999)*. Myofibrillar fragmentation index (MFI) values were determined as described by *Hopkins et al. (2000)*.

Statistical analysis: Shear force measurements (kg), and MFI values were analysed using a mixed model with terms for stimulation, injection (inhibitors vs control), ageing (1 and 2 days), portion (cranial and caudal) and a random term of animal nested within stimulation treatment. First order interactions were also tested and discarded if not significant ($P < 0.05$). Predicted means were compared using the PDIFF statement.

Results.

There was a significant interaction between injection and ageing for shear force ($P < 0.05$; Table 1). As main effects both these factors also had a significant effect on shear force ($P < 0.001$), but there was no effect of stimulation, or portion, or of any first order interaction terms ($P > 0.05$). Injection and ageing had a significant ($P < 0.001$) impact on MFI values as did portion ($P < 0.05$; Table 1), with no significant effect of stimulation ($P > 0.05$). Interaction terms were not significant, although injection x portion approached significance ($P = 0.07$).

Discussion.

The results from both this study and an earlier study by *Hopkins et al. (1999)* showed that E-64 was effective at preventing tenderisation. In both our studies, administration of the inhibitor was very soon after death, when temperature, pH and the moisture

binding ability of the proteins were all high. Despite this *Uytterhaegen et al. (1994)* reported that when E-64 was injected at 24 hours post-mortem it still significantly decreased tenderisation. *Dransfield et al. (1992)* concluded that tenderisation due to the activity of calpains began when the pH fell to approximately 6.2, which if true implied that an inhibitor injected at 24 hours postmortem would not fully retard proteolysis. For this reason we opted to inject at or near death so the extent of proteolysis in the first 24 to 48 hours could be studied. Our data shows that E-64 increased toughness on average by 35% and in this experiment where shortening was in the normal range the shear force value of 7 kg could be considered the maximal level of toughness expected in lamb. If shortening was more severe then this level would rise. It is evident from the summary presented by *Dransfield (1999)* of studies where different types of inhibitors were used, that inhibitor concentration is an important factor. Delivery of the inhibitor to the intracellular environment will also impact on the response and it is argued that this explains why in the experiment of *Aalhus et al. (1996)*, only small differences in shear force between muscle injected with E-64 and control muscle were found. The authors acknowledged that permeability and diffusion of the compound might have influenced the results.

Our results showed that the cysteine proteases are responsible for post-mortem tenderisation. The decrease in proteolysis due to injection of E-64 was clearly seen in reduced MFI values, which to our knowledge is the first time this has been reported. Our results also suggested that the calpains, were the causative enzymes, since the use of an inhibitor specific to cathepsins B and L did not result in tougher meat than the controls. This would have been expected if these enzymes were active. We also confirmed that the inhibitor did prevent cathepsin activity when tested against a muscle homogenate *in vitro* (data not shown). 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 in which case they would be inactivated, given that the inhibitors must have reached the sarcoplasm as evidenced by the inhibitory effect of E-64. *Pommier et al. (1997)* suggested electrical stimulation may lead to physical disruption of lysosomal membranes. In addition *O'Halloran et al. (1999)* proposed that a faster rate of glycolysis results in an increased release of lysosomal enzymes and an increase in cathepsin B and L activity within the soluble fraction. However, if this had occurred in our study an interaction between stimulation and injection would have been expected and no interaction was evident in our data. Further study on the timing of the initiation of proteolysis is underway to clarify the factors that effect this important process as it impacts on final tenderness.

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Table 1. Predicted means (s.e.) for Warner-Bratzler shear (WB; kg) and MFI values for the significant main effects (injection, ageing, portion) and interaction (injection x ageing). *For injection A = E-64 and B = Z-Phe-Ala-CHN_2

Variable	Injection				Ageing			Injection x Ageing				
	Inhibitor - A	*Inhibitor - B*	*Control*	Av. s.e.	1	2	Av. s.e.	*Inhibitor - A*	*Inhibitor - B*	*Control*	*Ageing (days)*	Av. s.e.
WB	7.2a	5.5b	5.2b	0.25	6.3a	5.6b	0.23	7.3ax	6.0bx	5.7bx	1	0.28
								7.1ax	5.0by	4.6by	2	0.28
								Portion				
								Cranial	*Caudal*			
MFI	67a	82b	82b	2.99	72a	81b	2.48	74a	79b			2.50

Values followed by the same letter in a row (a, b) are not significantly different ($P = 0.05$) within main and interaction effects.
Values followed by the same letter in a column (x, y) are not significantly different ($P = 0.05$) within main and interaction effects.