Relationship between proteolytic changes and tenderness in prerigor lactic acid marinated beef

Per Ertbjerg,[1]* Martin M Mielche,[1] Lone M Larsen[2] and Anders J Møller[1]

[1]Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

[2]Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Abstract: A meat tenderising procedure involving injection of a lactic acid solution into prerigor muscle was investigated using beef *M pectoralis profundus*. The distribution of lysosomal enzymes in subcellular fractions, densities of myofibrillar protein bands after SDS-PAGE and shear force were measured in non-injected, 0.5 M and 1.0 M lactic-acid-injected samples during a 21 days ageing period. The activities of cathepsin B+L and β-glucuronidase in the soluble fraction increased with level of lactic acid and with time post-mortem ($P<0.001$). Lactic acid and storage decreased densities of SDS-PAGE bands migrating at the position of myosin heavy chain (MHC) and α-actinin and increased densities of a 150 kDa band ($P<0.01$). SDS-PAGE of isolated perimysium cleaved with CNBr showed proteolytic cleavage of collagen after prolonged storage. Lactic acid injection significantly reduced shear force ($P<0.001$). The cathepsin B+L activity in the soluble fraction correlated to shear force ($r=-0.8$), the degradation of MHC and α-actinin ($r=-0.88$ and -0.90) and the generation of the 150 kDa fragment ($r=0.90$) but not to the generation of a 31 kDa fragment ($r=0.05$). A major part of the tenderness improvement after lactic acid injection was complete at 24 h post-mortem, and was therefore due to a rapid process, eg pH-induced swelling of the muscle structure. The data on enzyme activities and protein degradation, however, suggested that the action of lysosomal cathepsins also contributed to textural changes.

Keywords: acid marination; lysosomal integrity; tenderness; cathepsin; proteolysis

INTRODUCTION

Meat tenderness is well known to be highly influenced by temperature and pH during rigor development in muscle post-mortem (pm). The effect of rigor temperature is most clearly observed in prerigor excised muscle strips in which the shortening–tenderness relationship is well established. Recently a temperature region of 7 to 15 °C during rigor was shown to result in minimum shortening and least toughness for the beef muscles *M longissimus dorsi* and *M semimembranosus*.[1,2] The rate of pH fall however, has been reported to affect tenderness by mechanisms other than muscle shortening.[3] This probably reflects the complex nature of the pH-induced effects which, in combination with muscle temperature, influence swelling, proteolysis and shortening in the early rigor phase.

Artificial tenderisation by acid marination, ie the soaking of meat in an acidic solution, is a commonly used culinary technique. The tenderising effect is of particular commercial interest for upgrading tougher and cheaper cuts of a carcass. The potential causes for tenderisation due to marination are: (a) pH induced swelling of muscle fibres and/or connective tissue; (b) accelerated or additional proteolytic weakening of muscle structure, and (c) increased solubilisation of collagen upon cooking.[4]

From a series of experiments aimed to identify the relationship between pH and tenderness, Gault [5,6] showed that toughness increased when the pH of meat was decreased from 5.5 to 5.0 but a dramatic reduction in toughness occurred when the pH was reduced below 5.0, particularly in the range 4.6 to 4.1. The tenderisation observed at pH values below pH 5.0 was mainly believed to be caused by the increased swelling of raw meat and is in close resemblance to the described influence of acidic pH on the water-holding capacity of ground meat.[7] The lowering of meat pH is also favourable for an increased release and activity of lysosomal cathepsins.[8] This may cause an enhanced potential for proteolysis as the lysosomal enzymes cathepsin B and L can cleave both myofibrillar proteins[9,10] and collagenous proteins.[11,12]

A more recent concept of marination involved injection of lactic acid into muscle prerigor to achieve both an earlier post-mortem activation of muscle cathepsins and an accelerated, more even distribution

* Correspondence to: Per Ertbjerg, Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

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of acid throughout the meat.[13] Injection of bovine muscle with 0.1 M lactic acid immediately after slaughter to a level of 10% of the original weight caused rapid pH decline in prerigor meat (minimum pH 5.33 after 3 h post-mortem) and increased degradation of perimysial collagen. The increased proteolysis was most probably due to an earlier activation of cathepsins as Ertbjerg *et al*[8] showed both enhanced and earlier post-mortem release of cathepsins when muscle pH was decreased to 5.1 by a similar procedure for lactic acid injection. Eilers *et al*[14] injected a 0.3 M lactic acid solution into hot boned beef muscle which significantly reduced Warner–Bratzler shear force compared to cold boned and hot boned controls.

The objective of the present work was to provide more insight into the biochemical role of tenderisation caused by prerigor injection of lactic acid. We examined the release of lysosomal enzymes, the degradation of myofibrillar and collagenous proteins by SDS-PAGE and tenderness as measured by Warner–Bratzler shear force. The role of proteolysis in tenderisation of lactic acid marinated meat is discussed in light of the relationship between the measured traits.

MATERIALS AND METHODS

Muscles

Bovine *M pectoralis profundus* were excised from both sides of six Black Pied Danish cows (3 to 4 years old) within 30 min of slaughter. The muscle was trimmed for visible connective tissue and fat. Triangled areas at anterior and posterior ends and thinner areas at dorsal and ventral parts were removed. Twelve samples of 8×12 cm were obtained from each animal. Shear force, cooking loss and pH were measured on all samples, enzyme activities and SDS-PAGE of myofibrils were performed on samples from two animals and SDS-PAGE of perimysial collagen on samples from four animals.

Lactic acid injection

Muscle samples (*c* 2.5 cm thick, 200–300 g) were either non-injected controls or injected with 0.5 or 1.0 M lactic acid to a level of 10% of the original weight using a multi-pipette (Eppendorf 4780 with a Plus/8 adaptor) with fixed needles. Injections were performed in three depths (50 µl depth^{-1}) with 0.5 cm between each needle injection point. Injected lactic acid was pretempered to 15 °C. After injection the meat was placed at 15 °C for 24 h and then vacuum packed before storage at 4 °C for 1, 7, 14 or 21 days. At selected times post-mortem, pH was measured using a direct insertion probe electrode (Ingold Lot 406-M3).

Homogenisation and subcellular fractionation

Homogenisation and subcellular fractionation were performed as previously described.[8] Briefly, samples (1.5 g) were homogenised using a Potter-Elvehjem-type homogeniser and subcellular fractionated to a myofibrillar fraction ($1100 \times g$ for 10 min), a membrane fraction (the combined activity in pellets after $3000 \times g$ for 10 min; $27\,000 \times g$ for 20 min and $100\,000 \times g$ for 60 min) and a soluble fraction (the final supernatant). Duplicate fractionations were carried out.

Cathepsin B+L and β-glucuronidase assays

Enzyme activities in fractions were assayed fluorimetrically using the substrate N-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarine for cathepsin B+L as described[15] and 4-methylumbelliferyl-β-D-glucuronide for β-glucuronidase.[16] Activities are shown as µU, where 1 µU is defined as the release of 1 pmol of product per min.

Isolation of myofibrils

Myofibrils were prepared as previously described[17] and protein concentration was determined by the biuret procedure[18] using bovine serum albumin as standard. Samples were prepared for SDS-PAGE by dissolving myofibrils in sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.32 M dithiothreitol and 0.0025% (w/v) bromophenol blue).

Isolation of insoluble perimysial collagen

Insoluble perimysial collagen was prepared by a modification of the method described by Stanton and Light.[19] The meat sample (50 g) was divided into pieces of less than 1 g and then homogenised in 25 ml 0.05 M $CaCl_2$ (ice-cooled) for 15 s in a Waring Blender. The homogenate was filtered through a graded copper grid (1 mm^2 perforations) and material retained on the filter was re-homogenised in 25 ml 0.05 M $CaCl_2$ and re-filtered. This process was repeated a further six times, using 25 ml 0.05 M $CaCl_2$ each time. The retained material was then stirred in 25 ml 6 M urea, 0.05 M tris-HCl, pH 7.4 for 30 min on a magnetic stirrer, then centrifuged at $4300 \times g$ for 10 min. A further 25 ml of buffered urea were added to the aggregated insoluble fraction and the extraction procedure repeated twice. The remaining insoluble perimysial fraction was dialysed against five changes of water for 16 h and then frozen subsequently and freeze-dried.

CNBr digestion of insoluble perimysial collagen

The freeze-dried perimysial material was cut into minor pieces with scissors and further processed into very small pieces using an Ultra-Turrax homogeniser for 6×5 s (without added buffer). The dry material was suspended in 70% (v/v) formic acid to a concentration of 10 mg ml^{-1} and CNBr cleavage was then performed essentially as described by Light.[20] Freeze-dried CNBr-cleaved peptides were dissolved at a concentration of 10 mg ml^{-1} in SDS sample buffer and subjected to SDS-PAGE. Peptide bands were identified by comparison with previous results.[19]

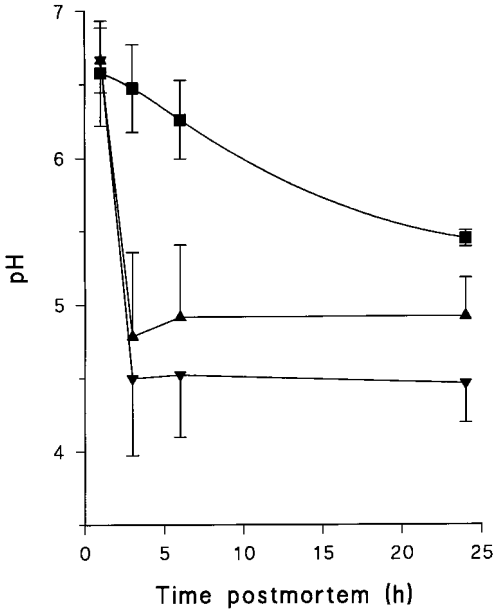

Figure 1. Muscle pH decline to 24 h post-mortem. ■, untreated control; ▲, 0.5 M lactic acid; ▼, 1.0 M lactic acid. Means ± SD are shown.

SDS-PAGE

Electrophoretic procedures of Laemmli[21] were followed. Each sample was run in duplicate using tris-glycine precast gels (Novex). Myofibrillar proteins were separated using discontinuous 8 to 16% acrylamide gradient slab gels with 2.6% (w/v) bisacrylamide. Perimysial collagen peptides were separated on 14% (w/v) acrylamide gels containing 2.6% (w/v) bisacrylamide. Densitometric scans of gels were performed using the CREAM scanning system (Kem-En-Tec Software Systems, Denmark). To standardise image analysis procedures, the baseline was defined by the computer software (low level background correction). Peak area values of SDS-PAGE bands were determined and expressed as percentage of total peak area of all bands in the sample.

Cooking loss and shear force

Vacuum-packed samples were heated for 120 min at 60 °C. Cooking loss was determined by weighing samples before and after heating. Warner–Bratzler (WB) shear force ($N\,cm^{-2}$) measurements were performed as described by Møller.[22] Each sample resulted in 12 WB deformation curves for calculating mean values. Two parameters were measured: WB *M*-force (initial yield) and WB *C*-force (final yield) taken as an indication of the myofibrillar and connective tissue components of tenderness, respectively.

Statistical methods

Effects of animal, time post-morterm and level of lactic acid injection were examined by standard analysis of variance in a factorial block design with animals as blocks. Enzyme activities and densities of protein bands were examined separately for each subcellular fraction. Prior to analysis of variance, the enzyme activities were transformed into logarithms to stabilise

Table 1. P-values obtained after analysis of variance

	Animal	*Storage time*	*Level of lactic acid*	*Storage time × lactic acid*
Cathepsin B+L activity				
Soluble fraction	0.27	<0.001	<0.001	0.34
Membrane fraction	0.04	<0.001	<0.001	0.03
Myofibrillar fraction	0.35	<0.001	<0.001	0.28
β-glucuronidase activity				
Soluble fraction	<0.001	<0.001	<0.001	0.02
Membrane fraction	<0.001	<0.001	<0.001	0.57
Myofibrillar fraction	<0.001	<0.001	0.001	0.19
Myofibrillar protein bands				
MHC	0.16	0.002	<0.001	0.95
150 kDa	0.57	<0.001	<0.001	0.39
α-actinin	0.07	0.01	<0.001	0.51
95 kDa	0.08	0.35	0.003	0.12
31 kDa	0.02	<0.001	0.003	0.13
Perimysial protein bands				
>200 kDa	0.001	0.01	0.98	0.14
α-2(I)CB3,5	0.006	0.18	0.16	0.34
α-1(I)CB3-7	0.04	0.83	0.48	0.85
α-1(I)CB7	0.009	0.74	0.44	0.76
α-1(I)CB8	0.008	0.21	0.40	0.28
0–4 kDa	0.27	0.009	0.23	0.05
Shear force				
WB *M*-force	<0.001	0.05	<0.001	0.15
WB *C*-force	0.04	0.61	<0.001	0.98
Cooking loss	0.008	0.23	<0.001	0.90

Table 2. Activity of cathepsin B+L and β-glucuronidase ($\mu U\,g^{-1}$ muscle) in the soluble, membrane and myofibrillar fraction at 4 h, 1 d, 7 d and 21 d post-mortem, in non-injected (NI), 0.5 and 1.0 M lactic-acid-injected samples

		Cathepsin B+L				*β-glucuronidase*			
Fraction	*Time post-mortem*	*NI*	*0.5M*	*1.0M*	*Average**	*NI*	*0.5M*	*1.0M*	*Average**
Soluble	4 h	489	658	1060	669a‡	613	622	833	663a
	1 d	758	1420	1970	1266b	716	1220	1170	984b
	7 d	1370	2050	2820	1981c	1150	1710	1740	1436c
	21 d	1700	2950	3100	2445c	1500	1640	1720	1574c
	average†	957a	1600b	2139c		905a	1218b	1316b	
Membrane	4 h	1230	920	836	991a	1020	725	464	721a
	1 d	931	660	359	584b	402	294	124	239b,c
	7 d	1070	589	309	565b	426	278	108	205c
	21 d	972	760	475	694b	569	326	177	301b
	average†	1032a	695b	426c		539a	323b	165c	
Myofibrillar	4 h	229	179	145	183a	301	307	262	284a
	1 d	275	232	186	224a,b	402	339	253	321a
	7 d	390	281	196	273b	628	605	462	552b
	21 d	310	235	132	210a	718	553	610	608b
	average†	291a	227b	162c		473a	431a,b	375b	

* Antilog[average log(enzyme activity)] at each lactic acid treatment.

† Antilog[average log(enzyme activity)] at each storage time.

‡ Means of cathepsin B+L or β-glucuronidase in the same column or row with different following letters differ significantly ($P<0.05$).

variation of data. For comparison of means, Tukey's method was used. All statistical calculations were carried out using SAS STAT™ software.

RESULTS

Temperature and pH

Samples were injected at approximately 1 h post-mortem with lactic acid pretempered to 15 °C. Muscle temperature was 27 ± 4 °C at time of injection. Non-injected samples reached a pH of 5.44 within 24 h (Fig 1). The pH of lactic-acid-injected samples decreased rapidly, and seemed to have stabilised in less than 2 h after injection. At 24 h, samples injected with 0.5 or 1.0 M lactic acid had mean pH values of 4.92 and 4.45, respectively.

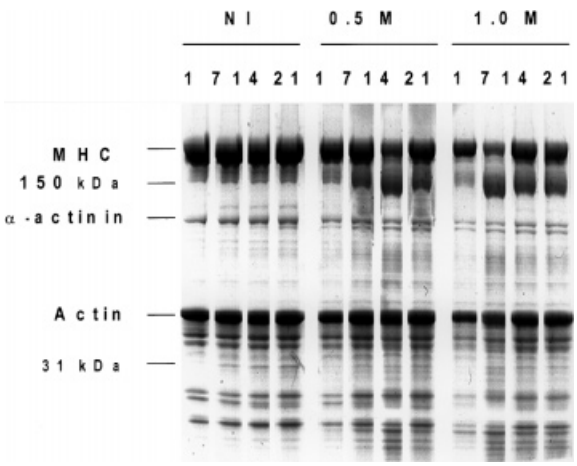

Figure 2. SDS-PAGE of myofibrillar proteins. NI, non-injected samples stored 1, 7, 14 and 21 days; 0.5 M, injected 0.5 M lactic acid stored 1, 7, 14 and 21 days; 1.0 M, injected 1.0 M lactic acid stored 1, 7, 14 and 21 days; MHC, myosin heavy chain.

Lysosomal enzymes

Activities of cathepsin B+L and β-glucuronidase of all subcellular fractions were significantly ($P<0.001$) affected by main effects of storage time and level of lactic acid injection (Table 1). The interaction between storage time and lactic acid level significantly affected only activities of cathepsin B+L in the membrane fraction and β-glucuronidase in the soluble fraction ($P<0.05$).

In the soluble fraction average cathepsin B+L activities increased ($P<0.05$) with increased level of lactic acid injection (Table 2). Average cathepsin B+L and β-glucuronidase activities also increased significantly with increased storage time up to 7 days. Mean cathepsin B+L activities in non-injected and lactic-acid-injected samples increased more than threefold from 4 h to 21 d post-mortem. Changes in cathepsin B+L and β-glucuronidase activities in the membrane fraction tended to be opposite to those seen in the soluble fraction and thus decreased ($P<0.05$) with increased level of lactic acid but with no effect of storage beyond 1 day. The myofibrillar fraction showed a tendency to increased lysosomal enzyme activities with storage time and decreased activities with lactic acid injection.

SDS-PAGE of myofibrillar proteins

Myofibrillar protein changes are shown on the gel in Fig 2. Analysis of variance (Table 1) showed that the level of lactic acid significantly affected the densities of all measured protein bands ($P<0.01$) while storage time affected the densities of all protein bands ($P<0.05$) except the 95 kDa band. Interactions between storage time and lactic acid level were not significant.

Average myosin heavy chain (MHC) densities

Protein band	Time post-mortem (days)	NI	0.5M	1.0M	Average*
MHC	1	31.0	27.4	23.8	27.4a‡
	7	23.5	17.3	11.3	17.4b
	14	26.0	18.6	16.1	20.2b
	21	23.0	16.3	11.1	16.8b
	average†	25.9a	20.0b	15.6b	
150kDa	1	5.2	7.1	10.6	7.6a
	7	7.7	13.5	17.1	12.8b
	14	7.7	12.9	15.2	11.9b
	21	7.8	14.3	17.9	13.3b
	average†	7.1a	11.9b	15.2c	
α-Actinin	1	4.9	3.8	3.6	4.1a
	7	4.4	3.5	1.8	3.3a,b
	14	3.9	3.0	2.5	3.1b
	21	4.0	2.7	1.8	2.8b
	average†	4.3a	3.3b	2.4c	
95kDa	1	0.5	1.1	1.4	1.0a
	7	0.7	1.5	1.1	1.1a
	14	0.6	1.2	1.3	1.0a
	21	1.2	1.5	1.0	1.2a
	average†	0.8a	1.3b	1.2b	
31kDa	1	1.0	1.3	1.2	1.2a
	7	2.1	1.7	1.7	1.8b
	14	2.4	1.5	1.7	1.9b
	21	3.1	2.0	1.8	2.3b
	average†	2.2a	1.6b	1.5b	

Table 3. Effect of lactic acid injection during ageing on the density of myosin heavy chain (MHC), 150kDa band, α-actinin, 95 and 31kDa bands. NI: non-injected samples. The density of each protein band is expressed as percentage peak area of total peak area from all stained proteins in the lane

* Average density of protein bands at each storage time.

† Average density of protein bands at each lactic acid treatment.

‡ Mean densities in rows or columns within each protein band with different following letters differ significantly ($P<0.05$).

decreased significantly ($P<0.05$) in lactic-acid-injected samples and with storage from day 1 to day 7 (Table 3). Concomitant with decreased MHC densities a major degradation product appeared at approximately 150kDa. Average densities of the 150kDa band increased ($P<0.05$) with increasing lactic acid level and with storage from day 1 to day 7. Average densities of the α-actinin band decreased ($P<0.05$) with increasing lactic acid level and with storage time from day 1 to day 14. Average densities of the 95kDa band increased significantly ($P<0.05$) in lactic-acid-injected samples but were unaffected by storage. Average 31kDa densities decreased in lactic acid-injected samples but increased ($P<0.05$) with storage time from day 1 to day 7. The appearance of the band at 31kDa was most clearly seen during storage of non-injected samples (Fig 2 and Table 3).

SDS-PAGE of intramuscular connective tissue

Perimysial collagen from non-injected and lactic-acid-injected meat aged 1 or 21 days was isolated and cleaved by cyanogen bromide. No significant effect of lactic acid injection on perimysial collagen was seen after SDS-PAGE analysis (Tables 1 and 4). Some intensifying of material in the very low molecular weight region with time post-mortem was seen, however (Fig 3). Densitometry of gels showed increased ($P<0.05$) density of the band migrating with an M_r of approximately 4kDa in non-injected samples during storage (Table 4) and at the same time the first major band ($M_r>200$kDa) decreased ($P<0.05$). Results showed no significant changes in other investigated peptide bands.

Table 4. Effect of 1.0M lactic acid injection and ageing on the density of peptides from CNBr cleaved perimysium. The density of each protein band is expressed as percentage peak area of total peak area from all stained proteins in the lane

	Non-injected		1.0M lactic acid injected	
Peptide band	1day	21days	1day	21days
>200kDa	24.9a*	20.6b	23.5a,b	22.1a,b
α-2(I)CB3,5	14.6	12.9	12.9	12.5
α-1(I)CB3-7	6.3	6.0	6.6	6.5
α-1(I)CB7	8.4	8.7	9.0	9.0
α-1(I)CB8	8.4	8.3	9.1	8.2
0–4kDa	6.6a	12.6b	10.5a,b	11.5b

* Means ($n=4$) in the same row with different following letters differ significantly ($P<0.05$).

Shear force

Lactic acid injection reduced ($P<0.001$) WB *M*-force and WB *C*-force (Table 1). Injection of 1.0M lactic acid reduced WB *M*-force and *C*-force to approximately half the values as obtained for non-injected samples, and with intermediate shear force after 0.5M

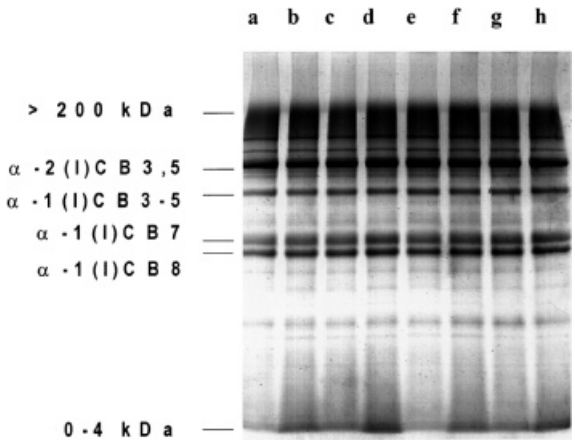

Figure 3. SDS-PAGE of CNBr peptides of insoluble perimysial material. Lane a and e, non-injected, 1 day; lane b and f, non-injected 21 days, lane c and g, injected 1.0 M lactic acid, 1 day; lane d and h, injected 1.0 M, 21 days.

lactic acid injection (Fig 4). The tenderising effect of lactic acid was seen at 1 day post-mortem and with no significant change during further storage while tenderisation in non-injected controls appeared as a gradual decrease in WB *M*-force during the storage period.

Cooking loss

Cooking loss for control samples and lactic-acid-injected samples were unaffected ($P > 0.1$) by storage time, but affected ($P < 0.001$) by level of lactic acid (Table 1). Mean percentages of cooking loss were approximately 23% for non-injected samples, 32% for 0.5 M and 28% for 1.0 M lactic-acid-injected samples.

Correlation between traits

As shown in Table 5, cathepsin B+L activity in the soluble fraction was significantly correlated to degradation of MHC ($r = -0.88$), generation of 150 kDa fragment ($r = 0.90$) and the degradation of α-actinin ($r = -0.90$) but not correlated to generation of 31 kDa fragment ($r = 0.05$). Shear force values were correlated to cathepsin B+L activities in the soluble and the membrane fractions and the degradation of MHC and α-actinin. Appearance of the 31 kDa peptide was, for non-injected samples, correlated ($r = -0.87$) to WB *M*-force (results not shown), but not significantly correlated when data from lactic-acid-injected samples were included (Table 5).

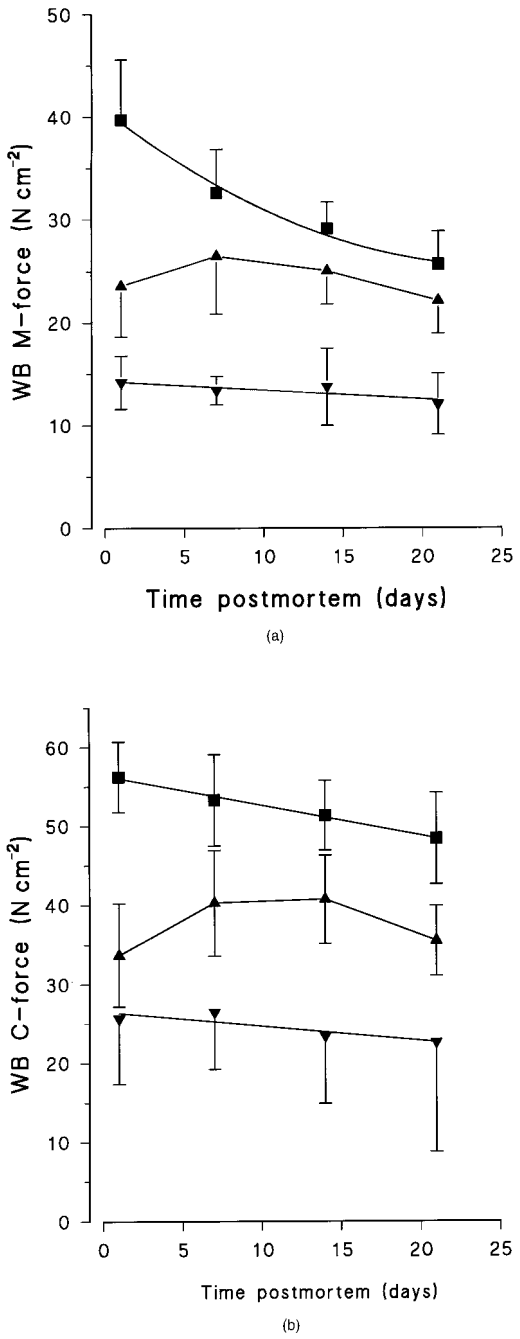

Figure 4. (a) Warner–Bratzler *M*-force and (b) Warner–Bratzler *C*-force, during ageing of lactic-acid-injected bovine *pectoralis profundus*. ■, untreated control; ▲, 0.5 M lactic acid; ▼, 1.0 M lactic acid. Means ± SEM ($n = 6$) are shown.

DISCUSSION

In the work presented here, we examined the involvement of proteolysis as part of the tenderisation mechanism in prerigor lactic acid marinated beef. A low temperature during rigor may cause toughening, but also a combination of fast pH decline to approximately 5.5 at 2 h post-mortem and high temperature resulted in lowered meat tenderness in beef *M longissimus dorsi*.[3] Increased susceptibility of calpains to autolysis and denaturation at pH of approximately 5.5 in combination with high temperature was suggested to have caused the increased toughness. In the present experiment a temperature of 15 °C during rigor was intended by injecting lactic acid tempered to 15 °C and by storage of samples at that temperature until 24 h post-mortem. At the time of lactic acid injection, however, the muscle temperature was approx. 27 °C. This combination of a relatively high temperature and muscle pH lower than 5.0 after lactic acid injection is likely to have caused extensive loss of calpain activity, as Simmons *et al*[23] showed a rapid decrease of calpain activity with decreasing pH to 5.5 at high rigor temperatures in

Table 5. Correlation coefficients between activity of cathepsin B+L in the soluble and membrane fraction, band density after SDS-PAGE of myofibrillar proteins and Warner–Bratzler shear force[a]

	Cathepsin B+L membrane fraction	*MHC*	*150 kDa*	*α-actinin*	*95 kDa*	*31 kDa*	*WB M-force*	*WB C-force*
Cathepsin B+L soluble fraction	−0.63***	−0.88***	0.90***	−0.90***	0.30	0.05	−0.79***	−0.80***
Cathepsin B+L membrane fraction		0.55*	−0.69**	0.58*	−0.37	0.32	0.79***	0.82***
MHC			−0.91***	0.73***	−0.43*	0.16	0.76***	0.82***
150 kDa				−0.87***	0.46*	−0.08	−0.83***	−0.86***
α-actinin					−0.32	0.17	0.71***	0.67***
95 kDa						0.12	−0.55**	−0.51*
31 kDa							0.14	0.27
WB *M*-force								0.77***

[a] Measurements from samples with common treatment (1, 7, 14 and 21 days post-mortem for non-injected, 0.5 M and 1.0 M injected) are used. $n = 18$, except for correlations between cathepsin B+L activities in the soluble and membrane fraction ($n = 24$) and the correlation between WB *M*-force and WB *C*-force ($n = 72$). The level of significance is denoted by *,** and *** for significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

beef *M longissimus dorsi*. As the pH values were below 5.0 in the present study, calpain activity was not considered any further, and only the effect of lower pH on lysosomal proteolytic enzymes was determined. In agreement with our previous results,[8] prerigor lactic acid injection resulted in significantly increased activities of cathepsin B+L and β-glucuronidase in the soluble fraction and decreased activities in the membrane fraction, indicating that enzymes were released from the lysosomes. The measured activities of cathepsin B+L reflects their proteolytic potential while the β-glucuronidase measurements serve as a more general indicator of lysosomal enzyme release as no known endogenous inhibitor against β-glucuronidase exists.

Increased level of lactic acid resulted in significantly increased myofibrillar protein degradation as indicated by intensity changes in SDS-PAGE bands (Fig 2 and Table 3). The most pronounced changes were decreased MHC band intensity and concomitant appearance of a band at approximately 150 kDa. Also the α-actinin band intensity clearly decreased after lactic acid injection. Previous studies suggest that cathepsins are likely to have caused these protein degradations. Isolated MHC is susceptible to attack from cathepsins D, B and L.[24–26] Also incubation of myofibrils with cathepsin D, B or L[9,10,26] or lysosomal extract[10,27] resulted in MHC degradation and appearance of bands in the size range 120 to 160 kDa. Cathepsin L can, at pH 5.0, degrade the Z-disk protein α-actinin to peptides of slightly lower M_r values and with further degradation at lower pH values of 3.0 to 3.5.[26] MHC and α-actinin has been reported to be degraded in isolated myofibrils at pH below 4.8, and the involvement of cathepsins was suggested.[28] Finally, the pH of lactic-acid-injected samples is within the range of optimum pH of cathepsin B and L activity. These observations all suggest that the lactic acid induced degradation of MHC and α-actinin in our study is caused by lysosomal enzymes such as cathepsins D, B or L. This conclusion is further supported by the high correlation (Table 5) between cathepsin B+L activity in the soluble fraction and the density of MHC ($r = -0.88$) and α-actinin ($r = -0.90$).

Storage for 7 days resulted in significantly increased average enzyme activities in the soluble fraction (Table 2), while the increase with further storage to 21 days was smaller and not significant. Protein degradations were mainly seen only from day 1 to day 7, where the average intensities of the MHC and α-actinin bands decreased and the average intensities of the 150 kDa and 31 kDa bands increased (Table 3). In contrast, the effect of lactic acid on tenderness was almost completed at day 1 (Fig 4) and therefore did not parallel the major proteolytic changes with storage. It can therefore be argued that the textural changes were too fast to be caused by the lysosomal enzymes. Lactic acid, however, had produced a considerable enzyme release from 4 h to 1 day, which may have been enough to induce sufficient proteolysis to cause the observed effect on tenderness at day 1. Another argument against an exclusive role of cathepsins in the tenderisation process comes from comparing the non-injected sample 7 days post-mortem and 0.5 M lactic-acid-injected samples 1 day post-mortem. The enzyme activities in the soluble fraction in these samples are in the same range and yet the shear force values are lower for lactic-acid-injected samples. The discrepancy in time-course changes between measurements relating to proteolysis and tenderness suggests that other tenderisation mechanisms were involved. Low pH induced swelling of muscle fibres and connective tissue or increased solubilisation of collagen during cooking are potential tenderisation causes in acid marination.[4,29] The role of swelling in the present study is supported by the observation that samples injected with 1.0 M lactic acid had lower cooking loss than the 0.5 M injected (28% versus 32%), suggesting that the former samples retained more liquid on cooking due to swelling. The increased tenderisation

by the lactic acid injection may therefore be caused by a combination of increased proteolysis and low pH induced swelling. Additional support for the involvement of lysosomal enzymes in the tenderisation caused by lactic acid injection appears from our earlier work using E-64 as an inhibitor of cysteine endopeptidases including cathepsin B and L. Injecting E-64 in combination with lactic acid (0.3 or 1.0 M) resulted in higher WB shear force values as compared to samples injected only with lactic acid.[30] These results suggest that proteolytic enzyme activity influences the tenderising effect of lactic acid and therefore strengthens the arguments for an involvement of lysosomal enzymes in the lactic acid tenderisation mechanism.

Storage caused a small degree of proteolytic cleavage in the perimysium as shown by two-dimensional SDS-PAGE.[31] In our study one-dimensional SDS-PAGE showed decreased intensity of the first major band ($M_r > 200$ kDa) and increased band intensity in the very low molecular weight region (0–4 kDa) after 21 days storage of non-injected samples (Fig 3 and Table 4), indicating a limited cleavage of perimysial collagen during storage. The small size in molecular weight of the generated peptide band suggests that the collagen molecules were cleaved from the ends.

In conclusion, prerigor lactic acid injection resulted in an earlier and enhanced release of lysosomal enzymes, increased degradation of myofibrillar proteins and increased tenderness. A significant relationship was seen between the release of cathepsin B + L activity from the lysosomes, changes in band intensities of MHC, α-actinin, a 150 kDa peptide and WB shear force values. Tenderness improvements after lactic acid injection were almost completed 24 h post-mortem, and were therefore, to a large extent, due to a rapid process. The tenderisation mechanism is concluded to involve a combination of pH-induced swelling of the muscle structure and increased proteolysis by lysosomal cathepsins.

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