EFFECT OF EPINEPHRINE AND EXERCISE ON PROTEOLYTIC ENZYME ACTIVITY IN PORCINE LONGISSIMUS MUSCLE

P. ERTBJERG[1], P. HENCKEL[3], A. KARLSSON[3], L. M. LARSEN[2] AND A. J. MØLLER[1]
[1]Dpt. of Dairy and Food Sci., [2]Chemistry Dpt., The Royal Vet. & Agric.Univ., Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark
[3]National Institute of Animal Science, Foulum, PO Box 39, DK-8830 Tjele, Denmark

BACKGROUND AND OBJECTIVE

High pH in porcine muscle occurs when muscle glycogen stores are depleted before slaughter, resulting in so called DFD meat. DFD is associated with increased tenderness, possibly arising from increased water holding properties producing laterally swelling (Tornberg, 1996). An additional explanation may be that high ultimate pH favours the action of the proteolytic enzyme μ-calpain. It can be hypothesized that low glycogen levels *ante-mortem* induce the calpain system to promote mobilisation of proteins as a muscle energy source. Increased *in vivo* protein degradation from the calpain system could be achieved either by increased calpain or decreased calpastatin levels, which again can lead to increased *post-mortem* proteolysis. The objectives of this study were to evaluate the effects of low energy levels at time of slaughter on calpain, calpastatin and cathepsin activity and on *post-mortem* proteolysis in porcine longissimus muscle.

METHODS

Animals were 10 crossbred pigs (Duroc sire and Large White-Landrace dam) from 5 litters (slaughter weight ca 90 kg). The muscle glycogen stores were depleted in five pigs by subcutaneous injection of epinephrine (0.3 mg/kg) at 10 to 15 h *ante-mortem* and exercise on a treadmill (5 min, 3.8 km/h) immediately before slaughter. Five siblings to the treated animals served as controls. Samples from *M. longissimus dorsi* (12 - 15th rib) was frozen in liquid nitrogen at 42 min, 24 h or 8 d post-mortem (pm) before storage at -80 °C. **Warner-Bratzler** (WB) shear force measurements were performed on vacuum-packed samples heated for 60 min at 80 °C as described by Møller (1981). Each sample resulted in 12 WB deformation curves for calculating mean values for max shear force. **Calpains and calpastatin** were separated by modification of the method described (Iversen *et al.*, 1993). Muscles were homogenised in 6 volumes of buffer (50 mM Tris/HCl, 5 mM EDTA, 10 mM monothioglycerol, 150 nM pepstatin A, pH 8.0). The supernatant after centrifugation was made 300 mM with NaCl and loaded on a phenylsepharose CL-4B column. Calpains were eluted by decreasing NaCl and loaded on a mono-Q column using a FPLC system. μ-Calpain and m-calpain were separated using a stepped NaCl gradient. Calpain activity was determined using casein as substrate by modification (volumes scaled down to 1/5) of the method described (Iversen *et al.*, 1993). One unit of calpain activity was defined as an increase in absorbance at 278 nm of 1.0 per hour at 25°C. A sample from the homogenate extract removed for **calpastatin determination** was heated at 100 °C for 3 min and centrifuged. The calpastatin activity was determined in an assay containing 0.2 units of m-calpain. One unit of calpastatin activity was defined as the amount that inhibited one unit of m-calpain activity. **Cathepsin B + L activities** were determined on non-frozen samples at 1 and 8 d pm by a slight modification of the described method (Ertbjerg *et al.*, 1998). A muscle homogenate was centrifuged at 1,100 *g* for 10 min (myofibrillar fraction); 20,000 *g* for 20 min (lysosomal fraction); 100,000 *g* for 60 min (microsomal fraction) and a soluble fraction (the final supernatant). The combined cathepsin B + L activities (1 mU = the release of 1 nmol of product per min) were determined fluorimetrically according to Kirschke *et al* (1983). **Myofibrils** were prepared as previously described (Møller *et al.* 1973). Myofibrillar proteins were separated by **SDS-PAGE** using 14% acryl amide slab gels. Densitometric scans of gels were performed and peak area values of bands were expressed as percentage of the actin band in the sample.

RESULTS AND DISCUSSION

The epinephrine and exercise treatment to deplete the energy stores resulted only in minor pH difference at 45 min pm while the ultimate pH was substantial higher (6.32 versus 5.66 in control). The treatment resulted in tender meat already at 1 d pm. Shear force values of treated animals were 43% lower ($P < 0.01$) at 1 d pm and 36% lower ($P < 0.05$) at 8 d pm (Table 1). In principle, the reduced toughness of the high ultimate pH group can be attributed either directly to the increased water holding capacity (e.g. water dilution of load bearing muscle elements after lateral swelling) or to increased muscle proteolysis. To define more accurately the nature of differing tenderness relating to the energy-depletion, we also measured the activities of muscle proteolytic enzymes that have been associated with tenderisation.

Table 1 shows that at 42 min and 24 h pm μ-calpain activity was 45 - 50% higher ($P < 0.05$) in the high ultimate pH group. With increased μ-calpain activity and unchanged calpastatin activity the overall proteolytic capacity of the calpain system would be expected to be increased. When the muscle energy stores are scarce *in vivo* then increased proteolysis by the calpain system may promote mobilisation of proteins as energy source. The 42 min calpain system determination is likely to reflect the *in vivo* level. The increased μ-calpain activity in energy-depleted muscles supports the hypothesis that a relationship between calpain proteolytic potential and muscle energy level exist in the living animal. The present study reporting increased μ-calpain activity in pre-slaughter energy depleted pig muscle contrasts results on beef by Geesink *et al.* 1992, where adrenalin treatment decreased μ-calpain activity at 29 h pm. Sensky *et al.* 1996 reported that epinephrine infused intravenously in pigs for a period of 1 week did not affect μ- and m-calpain at time of slaughter. During post-mortem storage only m-calpain showed consistent higher activities after epinephrine infusion, but the m-calpain activity in their study decreased rapidly with storage. Therefore the authors suggested that porcine longissimus muscle m-calpain is much less stable than m-calpain in tissues of ruminants. In contrast, in the present study analysis of variance showed that m-calpain activity was unaffected by storage ($P > 0.1$) as storage only decreased μ-calpain activity ($P < 0.01$) and calpastatin activity ($P < 0.05$). This agrees with studies on beef muscle reporting that during post-mortem storage μ-calpain and calpastatin decreases and m-calpain remains nearly constant (O'Halloran *et al.* 1997; Koohmaraie *et al.* 1987; Ducasting *et al.* 1985).

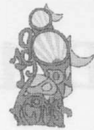

Table 1. Warner-Bratzler shear force (WB) at one and eight days post-mortem and calpain-system activity (U/g muscle) at 42 min and 24 h post-mortem as effected by epinephrine and exercise treatment (n = 5)

	WB (N)		μ-Calpain		m-Calpain		Calpastatin	
	1 d	8 d	42 min	24 h	42 min	24 h	42 min	24 h
Control	44.1	32.6	3.84	2.46	9.47	8.44	13.8	12.6
Treated	25.3	20.7	5.56	3.68	8.87	8.49	13.2	11.0
Significance	**	*	*	*	ns	ns	ns	ns

Treatment did not significantly affect cathepsin B + L activity in four subcellular fractions at 1 d but after 8 d storage the cathepsin B + L activity in the myofibrillar and the soluble fractions were lower ($P < 0.001$, Table 2). Reduced release of cathepsins from the lysosomes during storage may explain the lower cathepsin B + L activities in the soluble and myofibrillar fractions in high ultimate pH samples, although a corresponding increase in the lysosomal fraction was not observed.

SDS-PAGE after ageing showed significantly decreased intensity in treated samples of bands at 39, 37, 31 and 24 kDa (Table 3). The 31 and 24 kDa bands appeared during storage and were thus especially generated in controls. Part of the 31 kDa band seemed to be shifted to 30 kDa in treated samples. The results therefore show that the 39 kDa band were degraded during storage in treated samples having high ultimate pH, while some peptide fragments were preferentially generated in controls having normal ultimate pH. This suggests that different proteases played a dominating role in protein degradation in relation to meat pH. The calpain system (Table 1) would be expected to be very active and to dominate in the high pH samples. The cathepsins (Table 2) had higher free activity in the control samples and are known to have optimum activity at pH 5.5. Therefore the cathepsins are likely to contribute to the overall net muscle proteolysis at the normal ultimate pH, which might explain differences in protein degradation between control and treated samples.

Table 2. Cathepsin B + L activity (U/g muscle) in subcellular fractions at 1 and 8 days post-mortem (n = 3)

	Myofibrillar		Lysosomal		Microsomal		Soluble	
	1 d	8 d	1 d	8 d	1 d	8 d	1 d	8 d
Control	4.08	4.34	16.1	16.2	2.61	2.23	0.93	1.50
Treated	3.71	2.71	16.7	17.1	2.33	3.12	1.04	1.01
Significance	ns	***	ns	ns	ns	ns	ns	***

Table 3. Density of SDS-PAGE bands relative to actin (%) at 8 d post-mortem (n = 5)

	39 kDa	37 kDa	32 kDa	31 kDa	30 kDa	26 kDa	24 kDa	19 kDa
Control	27.3	34.5	4.1	5.5	4.2	20.9	12.4	29.4
Treated	19.5	29.9	3.6	1.5	7.6	21.5	2.9	26.4
Significance	***	*	ns	*	ns	ns	***	ns

CONCLUSIONS

Ante-mortem injection of epinephrine and treadmill exercise resulted in higher ultimate pH (6.32 versus 5.66 in control) and decreased ($P < 0.05$) shear force values. The muscle energy depletion treatment increased ($P < 0.05$) the muscle μ-calpain activity measured 42 min and 24 h pm by 45-50%, suggesting that a relationship between calpain proteolytic potential and muscle energy level might exist in the living animal. The high ultimate pH group showed lower ($P < 0.001$) cathepsin B + L activity in the myofibrillar and the soluble fractions after 8 d storage, suggesting that the increased ultimate pH increased the stability of the lysosomal membrane and by that reduced the release of cathepsins from the lysosomes during storage. SDS-PAGE showed that during storage a 39 kDa band were preferentially degraded in treated samples having high ultimate pH, while 32 and 24 kDa fragments were preferentially generated in controls having normal ultimate pH. Manipulating muscle glycogen level before slaughter may therefore provide additional insight in meat tenderisation as the changes in the pattern of protein degradation are likely to be caused by the changes in activities of the calpain and cathepsin systems.

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