ARE CATHEPSINS B AND D INVOLVED IN POST-MORTEM MYOFIBRILLAR DEGRADATION?

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Background

There is considerable evidence that the increase in tenderness that occurs during the post-mortem storage of muscle at 4°C is caused by limited proteolysis of the myofibril, although the detailed mechanism remains to be elucidated (Zeece et al. 1992, Jiang, 1998).
The principal proteinase systems in the muscle fibre are (a) the calpains, (b) the lysosomal cathepsins, and (c) the proteasome-ubiquitin system. Calpain seems to account for much of the post-mortem proteolysis in the myofibrils (Geesink and Koohmaraie, 1999), but the role of the cathepsins is unclear (Goll et al. 1983). The post-mortem activity of the proteasome has not been extensively investigated.
If cathepsins are to act post-mortem on the myofibril they must be released into the cytosol from the lysosome. Some studies, (e.g. Dutson, 1983) report post-mortem release of lysosomal enzymes, others (e.g. Lacourt et al. 1986) report no release. However, careful biochemical studies by Chambers et al. (1994) found limited release of lysosomal enzymes during the post-mortem storage of bovine muscle at 4°C and this finding was confirmed by Mobarak et al. (1999) using enzyme histochemistry and by Mobarak et al. (2000) using immuno electron microscopy.
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) can detect the subtle proteolysis that occurs post-mortem in myofibrils. The release of easily releasable myofilaments (ERM) from myofibrils is another sensitive test of post-mortem myofibrillar degradation. ERM are a sub-population of myofilaments on the myofibril surface that are easily shed from the myofibril under 'relaxing' conditions, i.e. 0.1M KCl, 3mM Mg-ATP, 3mM EGTA, 1mM DTT, 2-mM Tris-Maleate, pH 7.1. Reville et al. (1994) showed that the yield of ERM in beef muscle rises steadily over the conventional 14 day post-mortem ageing period of 14 days at 4°C.

Objectives

Assuming that lysosomal cathepsins are released into the cytosol post-mortem the present study investigated whether catheptic activity against myofibrils *in-vitro* mimics myofibrillar changes known to occur during post-mortem ageing of beef. The effect of pre-treatment of bovine myofibrils with cathepsin B or cathepsin D on ERM release from these myofibrils was examined. The proteolysis of the myofibrils by cathepsin B and cathepsin D was monitored by SDS-PAGE.

Materials and methods

Myofibrils were prepared from bovine sternomandibularis muscle (18 month old Friesian heifers) by the method of Etlinger et al. (1976) and stored in Low Salt Buffer (LSB) – 0.1MKCl, 3mM $MGCl_2$, 3mM EGTA, 1mM DTT, 20mM Tris-Maleate, pH 7.0 containing 50% glycerol at –20°C until required.
Cathepsin B treatment of myofibrils at pH 5.5: Bovine spleen cathepsin B and its inhibitor leupeptin were purchased from Sigma. The enzyme was reconstituted in 2mM phosphate buffer, pH 7.0 at a concentration of 1mg/ml. Myofibrils were resuspended in 0.1M KCl, 1mM EDTA, 50mM Imidazole, pH 5.5 at a protein concentration of 3mg/ml and digestion was started by adding cathepsin B at a ratio of 1 part enzyme to 100 parts myofibril (w/w). Two controls were employed – myofibrils incubated for 60 mins in the KCl, EDTA, Imidazole buffer, and myofibrils plus enzyme plus leupeptin incubated for 60 mins at 37°C. The active treatment conditions were myofibrils plus enzyme for 5 minutes or for 60 mins at 37°C. Treatment was terminated by the addition of leupeptin at a concentration of 20µg/ml.
Cathepsin D treatment of myofibrils at pH 5.5: Bovine spleen cathepsin D and its inhibitor pepstatin were purchased from Sigma. Cathepsin D, was reconstituted in distilled water, but otherwise the treatment of myofibrils with cathepsin D was effected as described for cathepsin B, except that 0.1M NaCl substituted for

0.1M KCl and digestion of myofibrils with cathepsin D was terminated by addition of pepstatin to a concentration of 20mM.

Assay of Mg-ATP Stimulated Release of Protein from Cathepsin Treated Myofibrils: Control and enzyme-treated myofibrils were resuspended in LSB and assayed for Mg-ATP stimulated release of protein in the ERM assay as described by Reville et al (1994). Released protein is expressed as a percentage of the total myofibrillar protein. Release of protein in the presence of LSB alone (i.e. no ATP) was also measured.

Electrophoresis by SDS-PAGE: SDS-PAGE was carried out on control myofibrils and on myofibrils treated with cathepsin D or cathepsin B. Myofibrils were solubilised in 4M urea, 1M thiourea, 0.025M Tris-Acetate pH 6.8, 37mM DTT, 1.5% SDS, 0.025% Bromophenol blue. Samples were heated at 55°C for 15 mins. and subsequently stored at –20°C until SDS-PAGE was performed.

SDS-PAGE followed the method of Laemmli (1970), using a 5% stacking gel and a 7%-18% linear acrylamide gradient separating get. 30µg of protein was loaded per lane. The gels were stained with coomassie-blue.

Results and discussion

Figure 1 shows that pre-treatment of myofibrils with cathepsin B or D enhanced Mg-ATP stimulated release of protein from myofibrils, even after 5 minutes pre-digestion of the myofibrils. In the case of cathepsin D pre-treatment, Mg-ATP stimulated release of protein rose from a mean of 1.3% total myofibrillar protein in controls, through a mean of 1.89% after 5 mins., to a mean of 5.0% after 1 hour's pre-digestion of the myofibrils. The corresponding figures for cathepsin B were a rise from 1.05% for controls, through a mean of 1.8% after 5 mins, to a mean of 3.1% after 1 hour's pre-digestion. Neither cathepsin D nor cathepsin B treatment of myofibrils had any significant effect on release of protein in the absence of Mg-ATP.

Figure 2 shows the protein subunit composition of control myofibrils and myofibrils after digestion for 5 mins. and 60 mins. with cathepsin B or cathepsin D at pH 5.5 as assessed by SDS-PAGE. Five minutes treatment with cathepsin B had little effect on the myofibril. Treatment of myofibrils for 1 hour with cathepsin B had pronounced effects, of which the most notable are:- most of the high molecular weight material above myosin is removed; myosin and actin bands are notably diminished compared with controls; many new bands, presumably degradation products, are evident, e.g. a ladder of bands in the range 120 kDa to 150 kDa relative molecular mass.

The effects of treatment of myofibrils with cathepsin D are similar to the results described for cathepsin B, although there are differences in the pattern of degradative products produced (Figure 2). Five minutes treatment at pH 5.5 shows but a minor effect on protein subunit pattern, most notably on the very high molecular weight sub-units. Sixty minutes treatment with cathepsin D shows a marked effect – a big diminution in very high molecular weight material, a marked decrease in myosin band intensity, and some degradation of α-actinin (not seen after cathepsin B digestion).

Increased ERM yields from cathepsin D or cathepsin B 60 minute pre-treated myofibrils compare favourably with the natural increase in ERM yield in post-mortem bovine muscle from 1.4% at-death to 5.5% after 14 days storage at 4°C. However, proteolysis of the myofibril during conventional ageing of meat is very minor and there is no degradation of actin or myosin, whereas 60 mins. digestion of myofibrils with cathepsin B or cathepsin D produces marked degradation of myofibrillar proteins, including actin and myosin. Therefore the effects of primary interest in the present study are the effects of limited (5 mins.) digestion of myofibrils by cathepsins.

Five minutes digestion of myofibrils by cathepsin B or D had only minor effects on the protein subunit composition of the myofibrils. This amount of pre-digestion of the myofibrils allowed an increase of about 75% in the amount of protein released from myofibrils in the presence of Mg-ATP compared to controls in the case of cathepsin B and 46% in the case of cathepsin D. If both enzymes were acting together, and if the effects were additive, this would produce about a 120% increase in the yield of releasable protein. If cathepsins are released from the lysosome post-mortem, other cathepsins, in addition to B and D, could also contribute to the degradation of the myofibril, e.g. cathepsin L.

Conclusions

Concerted but limited action of cathepsins on myofibrils post-mortem could account for much of the increase in Mg-ATP stimulated release of protein from myofibrils that is seen during the conventional post-mortem storage of beef at 4°C.

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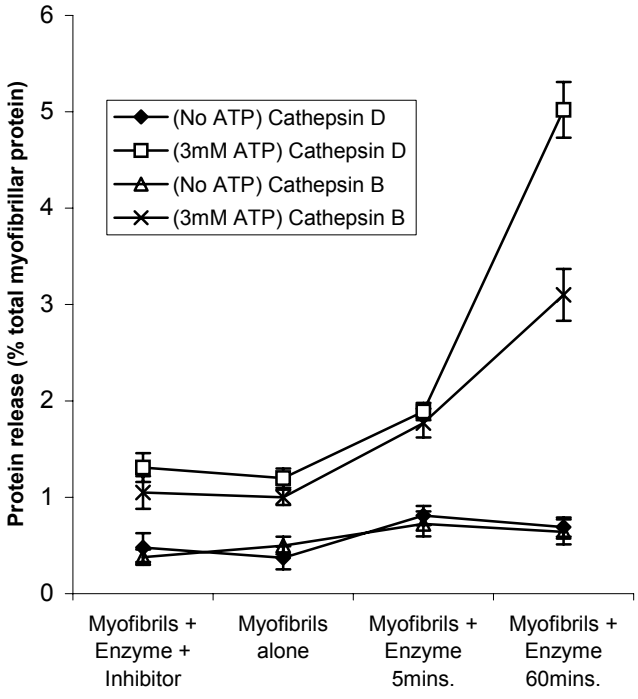

Figure 1 – Mg-ATP stimulated release of protein from cathepsin D or cathepsin B treated bovine myofibrils. Figures are means ± SD of releases from 5 animals.

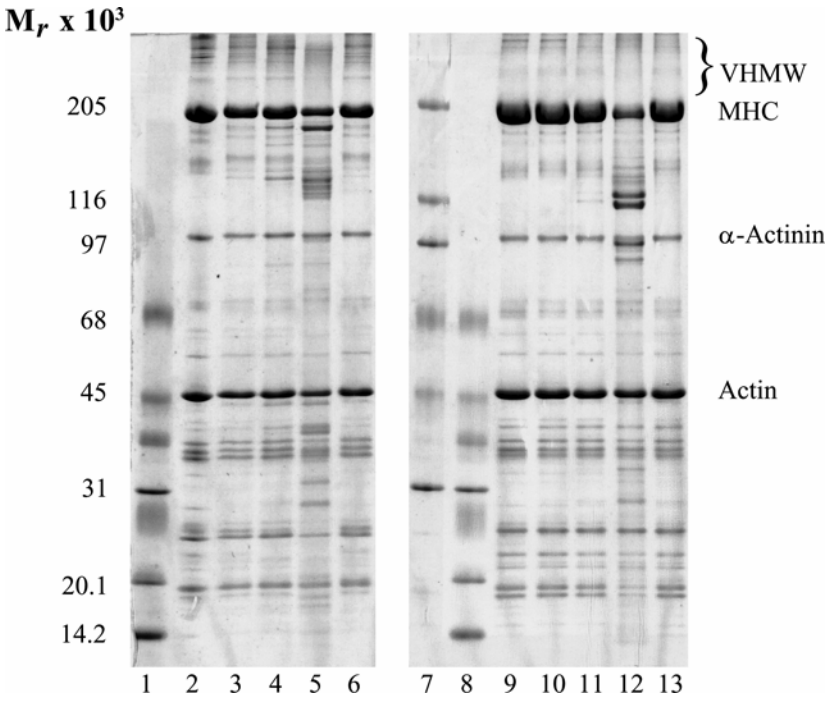

Figure 2 – Protein subunit composition of myofibrils as revealed by SDS-PAGE after treatment of the myofibrils with cathepsin B or cathepsin D at pH 5.5 at a cathepsin to myofibril ratio (w/w) of 1:100.
Lane 1: Low mol. wt. markers; Lane 2: Myofibrils from glycerol stock; Lane 3: Myofibrils held at pH 5.5, 1 hr., 37°C; Lane 4: Myofibrils + cathepsin B, pH 5.5, 5 mins. 37°C; Lane 5: Myofibrils + cathepsin B, pH 5.5, 60 mins, 37°C; Lane 6: Myofibrils + cathepsin B + Leupeptin, pH 5.5; Lane 7: High mol. wt. markers; Lane 8: Low mol. wt. markers; Lane 9: Myofibrils from glycerol stock; Lane 10: Myofibrils held at pH 5.5 for 1 hr. at 37°C; Lane 11: myofibrils + cathepsin D, 5 mins., pH 5.5, 37°C; Lane 12: Myofibrils + cathepsin D, 60 mins. pH 5.5, 37°C; Lane 13: Myofibrils + cathepsin D + pepstatin. VHMW = Very High Molecular Weight. MHC = Myosin Heavy Chain. M_r = Relative Molecular Mass.