Inactivation of μ-Calpain by EDTA

ERIC DRANSFIELD
Station de la Recherches sur la Viande, INRA de Theix,
Saint Genès Champanelle, 63122 France

BACKGROUND

The endogenous calpain-dependent proteinases, the calpains, are thought to be important in determining the tenderness of meat. Their activity *in situ*, however has not been measured and we can only speculate that the tenderising effect is due to their degradation of the cytoskeleton (Dransfield, 1997). Several factors are known to control their activity *in vitro*: pH, availability of calcium ions, temperature, their instability and the presence of their specific inhibitor, calpastatin. In living muscle, the free calcium level is thought to be about 0.1μM and too low to activate all the calpain. As rigor mortis develops, the concentration of free calcium ions rises to about 100μM and could activate the μ-calpain which is unstable and would decrease with storage time. Modelling the inactivation of μ-calpain gives a good prediction of the decline in toughness with storage (Dransfield, 1992). However, calpain autolysis post-mortem has been disputed (Geesink and Goll, 1995) and measured levels show that the decrease in μ-calpain is faster than in toughness (Zamora *et al.*, 1996) and did not correlate with tenderisation in electrically-stimulated beef (Geesink *et al.*, 1994). These differences may result from the use of different methods of measurement of μ-calpain, which is the subject of this paper.

OBJECTIVE

Determine the effect of EDTA and EGTA on calpain activity to aid the understanding of calpain decline in post-mortem muscle

METHODS

Calpain was extracted from beef *M. sternomandibularis* and the activity of μ-calpain measured continuously in the presence of ethylene diamine tetra acetate (EDTA), ethylene glycol-bis(ß-aminoethyl ether)N,N,N',N'-tetra acetate (EGTA) and insufficient and excess calcium chloride concentrations.

The μ-calpain and calpastatin: μ-calpain was partially purified from 400g of muscle by hydrophobic and ion exchange chromatography (Zamora *et al.*, 1996). Calpastatin was that in the supernatant after heating 2ml of extract for 95° for 2 minutes.

Measurement of calpain activity: The activity of μ-calpain was measured continuously using the fluorogenic substrate N-suc-leu-tyr-7amido,4methyl-coumarin (SLT; Sigma). Three millilitres of buffer A (50mM BisTris-HCl; 100mM KCl; 0.01% sodium azide; 0.1% Triton-X100; 10mM ß-mercaptoethanol; pH 7.5, 6.5 or 5.5) containing 250μM SLT and 0.4mM EDTA were placed in a 1cm spectroscopic cuvette and 100μl of enzyme extract was added. The fluorescence (wavelengths for excitation 350nm and for emission 460nm) was measured in a Perkin-Elmer fluorimeter using the time mode at room temperature. After a few seconds to record a stable baseline, 100μl of 50mM $CaCl_2$ in buffer A was added. After a period of time up to 30 minutes either 100μl of 100mM EDTA, followed a few minutes later by 100μl of 100mM $CaCl_2$ or the same quantities, but in the reverse order, were added. The fluorescence was monitored throughout.

RESULTS

Figure 1 shows 2 continuous records of the increase in fluorescence: one in which the EDTA solution was added before the calcium chloride solution and one when the EDTA was added after the calcium chloride solution.

Figure 1. Effect of calcium and EDTA on the activity of μ-calpain The increase in fluorescence was measured continuously in samples in which EDTA was added before or after $CaCl_2$.

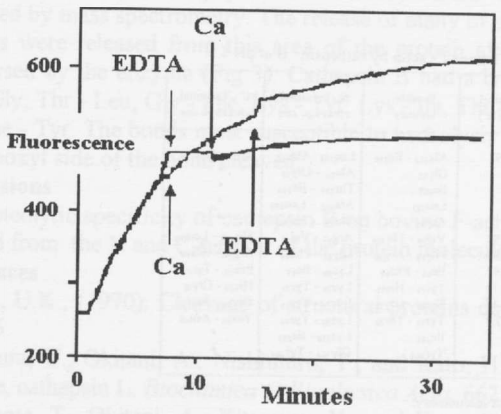
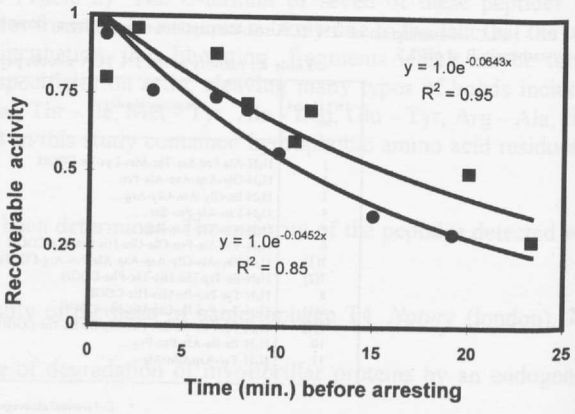


Figure 2. Decrease in activity of μ-calpain in relation to the time of EDTA (square) or EGTA (circle) addition. Recoverable act. is that after relative to that before EDTA



The sample to which calcium was added and followed by EDTA has always free calcium and, apart from the increase in fluorescence caused by the EDTA itself, shows a gradual asymptotic increase in fluorescence with time (Figure 1). When EDTA was added first,

the activity stopped and continued again after the addition of calcium. However, the rate after EDTA was lower than before the addition of EDTA and the total fluorescence (proteolysis) was less than when the Ca was added before EDTA. The effect of the time of addition of EDTA is shown in Figure 2. Later addition of EDTA gave lower recoverable activity and, when added at 12 minutes, only half the activity could be recovered. A similar effect was produced by EGTA (Figure 2). The effect was also observed at lower pH values (Figure 3). At pH 6.5 and 5.5, the recoverable activity after exposure to EDTA appeared to be less than at pH 7.5. The titration by calpastatin on the residual activity was unaffected by prior treatment with EDTA of EGTA (Figure 4).

Figure 3 Effect of pH on inhibitory effect of EDTA on the activity of μ-calpain. The line above each curve indicates addition of EDTA and below, $CaCl_2$

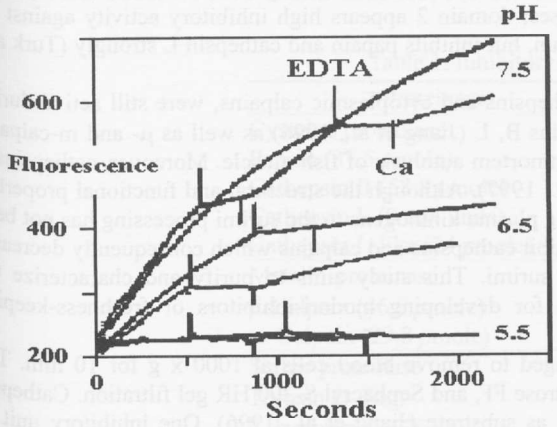
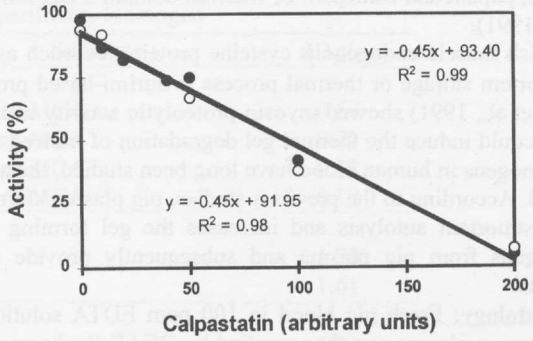


Figure 4 The inhibitory activity of calpastatin against activity of μ-calpain treated with EGTA (open circles) or with EDTA (closed circles).



DISCUSSION

During the extraction and purification of calpains, EDTA is used routinely to prevent activation of calpains by calcium ions and thus to stabilise the calpain. In meat research, a similar extraction procedure has been used not only for calpains in fresh muscle, in which little calpain is thought to be active, but also to follow the changes in calpains during the development of rigor mortis and throughout subsequent storage when calpain could be active. With an extraction procedure using EDTA, any active calpain would be underestimated considerably. This was examined in beef *M. Longissumus* stored at 12°C for 24 hours and then at 4°C, when it was found that the activity of partially-purified μ-calpain relative the number of active sites (assessed by back-titration with E-64) decreased from 66 in fresh muscle to 44 at 1 day and 41 at 2 days.

The mechanism of inactivation is not fully known but, in the presence of calcium ions, m-calpain is autolysed to at least 2 active forms. The first is converted to the second by internal cleavages which is then converted to the inactive form (Crawford *et al.*, 1987). The first form is reversibly inactivated by EDTA and is therefore measured by its activity on addition of sufficient calcium ion concentration. However, the 2nd form is irreversibly inactivated by EDTA and is not estimated by measuring activity. The rate of inactivation by EDTA, determined here with beef μ-calpain, was the same as that for chicken m-calpain (Crawford *et al.*, 1987). With this sequential autolysis, the effect of EDTA is temporal. In post-mortem muscle therefore, if calpain were activated, more loss would be induced by EDTA with increasing time post-mortem.

Using Western blotting also showed that losses may occur by non-extraction of calpain (Geesink and Goll, 1995) but the losses by non-extraction using routine extraction procedures were not quantified.

CONCLUSIONS

New methods should be sought to determine the active forms of calpain, particularly of μ-calpain, present in muscle and meat.

REFERENCES

Crawford, C., Willis, A.C. & Gagnon, J. 1987. The effects of autolysis on the structure of chicken calpain II. *Biochemical Journal*, **248**, 579-588.

Dransfield, E 1992. Modelling post-mortem tenderisation - III: Role of calpain I in tenderisation. *Meat Science*, **31**, 85-94.

Dransfield, E. 1997. When the glue comes unstuck. *43rd International Congress Meat Science and Technology*. Auckland, New Zealand. p. 52-61.

Geesink, G.H. & Goll, D.E. 1995. Measurement of calpain activity in post-mortem muscle extracts underestimates levels of μ-calpain. *41st International Congress Meat Science and Technology*. San Antonio, Texas. p. 547-549.

Geesink, G.H., van Laack, H.L.J.M., Barnier, V.H.M. & Smulders, F.J.M. 1994. Does electrical stimulation affect the speed of ageing or ageing response? *Sciences des Aliments*, **14**, 409.

Zamora, F., Debiton, E., Lepetit, J., Lebert, A., Dransfield, E. & Ouali, A. 1996. Predicting variability of ageing and toughness in beef *M. longissimus lumborum et thoracis*. *Meat Science*, **43**, 321-333.