IN VITRO MEASUREMENT OF CALPAIN ACTIVITY

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SUMMARY

Calpains play an important role in protein turnover in the cell during growth and development of the animal and could contribute significantly to the texture of meat. The *in situ* activity of calpains is presumed to be determined by their amount, their activation and inhibition, all of which change *post-mortem*.

The amount of calpains is normally assessed using casein as substrate but the uV-absorbing products from autolysis of calpain could interfere with the assay. Use of azo-casein and isothiocyanate-casein do not suffer this and were equally effective substrates.

Kinetic measurements of calpain activity often showed non-linear rates of product formation and that the amount of product formed was dependent of the temperature and was reduced at higher ionic strength. The rate of inactivation of m-calpain was lowered in the presence of calpastatin inhibitor.

Research would benefit from a standardisation of methodology.

INTRODUCTION

Calpain activity is said to play a role in protein turnover (Croall and DeMartino, 1991) and in meat texture (Dransfield, 1994). However the activity *in situ* has not been determined and is presumed to be a function of the level of calpains, calpastatin and the changes in activity which could occur during the conversion of muscle to meat and subsequent storage. *In vitro* determination of these reactions could be used to calculate the *in situ* activity (Dransfield, 1994).

Calpains and calpastatin are normally partially-purified and their amounts measured indirectly by *in vitro* assay. Calpains are activated by calcium ions and are optimally active at neutral pH. Such conditions are invariably chosen for assay. In the presence of calcium ions, calpains autolyse rapidly, producing forms of the enzyme capable of degrading exogenous substrates and these are then inactivated more slowly (Crawford *et al.*, 1987).

The total activity therefore depends markedly on the inactivation which is a function of the conditions, particularly the time and temperature of incubation, which differ considerably between laboratories. It is therefore appropriate to re-evaluate methodology for routine measurement of calpains.

EXPERIMENTAL

μ- and m-calpains were isolated from 40g fresh bovine muscle using the method similar to that of Etherington *et al.*, (1987) except that the separation of calpains was done on a DEAE-sephacel column using a step gradient of 250mM NaCl to elute a fraction containing μ-calpain and 500mM for m-calpain (about 15ml of each).

Purified m-calpain and calpastatin from human erythrocytes were a gift from Professor D.E. Goll's laboratory, Tucson, Arizona, USA.

Determination of proteolytic activity with caseins: 50mM Tris/HCl, 5mM dithiothreitol, 10 mg of casein/ml. and the solutions made to pH 7.5. The reaction was incubated for 20 minutes at 30°C and stopped by making the solutions 3% trichloracetic acid (TCA), cooled and centrifuged at 8000g for 3 min. Blank were made by addition of TCA at the start. Products in the supernatant using casein were assayed by absorption at a wavelength of 278nm; from azo-casein at 366nm; from FITC-casein by fluorescence with excitation at 490nm and emission at 525nm.

Kinetic of activity were measured using a synthetic fluorogenic substrate (suc-leu-tyr-NHMec from Bachem, Switzerland; where NHMec = 4-methyl-7-coumarylamide) using a similar buffer (3ml) with 250μM substrate, 0.1% Triton-X100, 0.1% azide and, normally, 100mM KCl. The fluorescence (excitation at 365nm and emission at 450nm) was measured continuously using a Perkin Elmer LS50B and data recorded every 10 seconds.
All the reactions were started by the addition of enzyme.

RESULTS

Assays using the caseins showed that the product was usually proportional to the amount of preparation used in the assay and that the amount of enzyme from 1g of muscle could be reliable assayed (Figure 1). With lower quantities of muscle the errors in detection increased. Slightly more consistent results were obtained with m- than with μ-calpain (Figure 1). All three caseins appeared equally effective.

The formation of product using suc-leu-tyr-NHMec was always non-linear. At 40°C rapid activity occurred within seconds but was completed in less than 10 minutes producing a low amount of product. Twice as much product was produced at 30° and activity ceased after 15 minutes due to inactivation of enzyme. The rate of inactivation decreased at 20°, 10° and increased at 0°C. Most proteolysis was produced at 15° to 20°C when the reaction was approximately linear for up to 1 hour at 20°C (Figure 2) and for more than 2 hours at 15°C. Variable results were obtained at 0° and at 40°C. A detailed examination at 20°C (Figure 3) shows that there was an initial lag period for 15 minutes and then an accelerated activity for upto 1 hour followed by a gradual decrease in activity, producing a sigmoid increase in product with incubation time (Figure 3).

In the absence of KCl in the incubation there was considerably more activity than with 250mM KCl (Figure 3). The rate of inactivation was little affected by the concentration of KCl.

At 30°C, calpastatin reduced the activity of m-calpain (Figure4). Increasing the concentration of calpastatin also reduced the rate of inactivation of calpain.

DISCUSSION

When calpains are activated by calcium ions proteolysis of calpain occurs and 2 active species have been isolated. Two further species may be present when EDTA is also present (Crawford *et al.*, 1987). This complexity poses particular problems for the analysis of calpains which are not evident, for example, with cathepsins which give linear increases in product with time.

The assays of calpain activity are often done at pH about 7.4 and between 20° to 40°C, usually with casein (5 to 10 mg/ml) as substrate and measuring the absorbance at 278nm of TCA soluble products (Dayton *et al.*, 1976). The activity may be standardised against the proteolysis produced by trypsin (Sarraga *et al.*, 1993), using Coomassie Brilliant Blue (Etherington *et al.*, 1987), against tryptophan (Uytterhaegen *et al.*, 1992) or as fluorescamine-positive material (Melloni *et al.*, 1982; Karlsson *et al.*, 1985). A variety of substrates have been used: denatured globin (Pontremoli *et al.*, 1990) provides a more sensitive substrate than casein (10 times as sensitive using fluorescamine). Purified myofibrils have also been used similarly (Reville *et al.*, 1976; Ouali and Valin, 1981; Koohmaraie *et al.*,1986; Cena *et al.*, 1992) measuring the absorbance of the supernatant at 268nm. Both activation and inactivation give rise to fragments of the enzyme, some of which are soluble in 3 to 5% TCA and which therefore could interfere with the assay. In practice, however, we could not detect any such effect at the levels used here which are likely to be in excess of those used in muscle and meat work.

Any such difficulties can be overcome by using a substrate which gives products which are detected in a different way to those from the calpains such as the substrates which give rise to products which are fluorescent (from fluorescein isothiocyanate-casein) or absorb in the visible region (from azo-casein). FITC-casein is 1000 times more sensitive than peptides measured at 280nm and more sensitive than fluorescamine (Twining, 1984, Wolfe *et al.*, 1989). With azo-casein, the TCA-soluble products are detected at about 366nm (Johnson & Guindon-Hammer, 1987) but can also give rise to acid soluble azo-casein peptides (Crawford *et al.*, 1993). ^{14}C-labelled azo-casein has recently been used (Takeuchi *et al.*, 1992).

After assay, the amount of product formed under standard conditions is then divided usually by the time of incubation, to produce a value quoted as an 'activity'. However, the data using suc-leu-tyr-NHMec showed that the activity was only constant at 15° to 20°C, temperatures which are rarely used. Similar findings were shown for succinylated lysozyme (5mg/ml) assaying the products using fluorescamine. At 30°C, the activity was reduced 30% after 10 minutes and 90% after 1 hour. At 37°C the formation of products was non-linear over 30 minutes, whilst at 21°C, the products increased linearly up to 40 minutes (Mellgren *et al.*

1982). Slightly more stablility was obtained with alkali-treated casein (5mg/ml) when proteolysis was linear up to about 1 hour at 21°C and linear for 10 minutes at 39°C (Karlsson *et al.* 1985). The rate of autolysis in the absence of substrate is faster (Koohmaraie, 1992; Geesink, 1993; Dransfield, 1994) but, after 2 hours at 5°C, 95% of the μ-calpain activity was retained at pH 7.0 and 80% at pH 5.8 (Koohmaraie, 1992) showing a strong temperature dependence of the stability. There is therefore a good evidence for employing 15° to 20°C for incubation rather than the 25° to 37°C used currently.

Measurements also depend on the ionic strength used in the assay and KCl may be added to prevent any precipitaion of calpain. Ionic strength increases *post-mortem* to about 300mM (Geesink, 1993) and differs amoung fractions eluted from columns using salt gradients. In this work, more than 90% of the activity was lost using 250mM KCl. This compares with a reduction to about 40% using casein as substrate (Geesink, 1993). This would suggest using lower salt concentrations than used currently either by dialysis or dilution of the sample. However, a slight increase in activity was observed up to 0.15M and then a gradual decrease up to 0.4M, also with casein substrate, and an increase in activity occurs using isolated beef myofibrils as substrate (Kendall *et al.*, 1993).

In this work, the initial rate of inactivation of m-calpain was reduced by 50% by a 3-fold weight of calpastatin. This may be due to the action of calpastatin which lowers of the concentration of the free calpain and inhibits only the later stages of breakdown of calpain (Mellgren *et al.*, 1982).

Furthermore, the rate of *in vitro* activity is then often quoted as a rate in the muscle or meat and shown to change with, for example, storage time of meat *post-mortem*. Both of these presentations could be misleading and should be avoided because they imply a direct relationship between the *in vitro* activity and the *in situ* activity which is independent of the time of sampling. This is unlikely to be the case in muscle or in meat soon after slaughter when the pH and temperature are changing. The description would be better represented as a decline in the level of calpains, albeit that the enzyme is assayed *in vitro* as an 'activity' and expressed in units of substrate consumed or product formed in a given time.

The rate of inactivation and the total proteolysis by calpains were markedly dependent on temperature in the range 0° to 40°C. Little activity was obtained at pH 7.0 at 0°C and at high temperatures. Karlsson *et al.* (1985) also remarked that no activity occurred at 0°C but no data were given. A low total amount of proteolysis at high temperatures has also been observed using casein as substrate but more proteolysis at pH 7.4 occurred at 5° than at 15°C, although the reverse was shown at low pH (Geesink, 1993).

Although the levels of calpains and calpastatin are poorly related to tenderness (Shakelford *et al.*, 1991), the *in vitro* results have shown that the rate of inactivation and activity of calpains are markedly dependent on temperature and pH in the range which occurs in meat *post-mortem*. The resulting variation in activity has been calculated to be the cause of much of the variation in the tenderness of meat (Dransfield, 1994).

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