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MEASUREMENT OF CALPAIN ACTIVITY IN POSTMORTEM MUSCLE EXTRACTS UNDERESTIMATES LEVELS OF μ-CALPAIN

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SUMMARY

μ-, and m-calpain were partly purified from bovine M. semimembranosus at 1 hour, 1 day, 2 days and 3 days postmortem. Western blots against the 28 kDa and 80 kDa subunits of the calpains showed little or no autolysis of extractable calpains at any time postmortem. Western blots against the 80 kDa subunit of μ-calpain showed an increased association of μ-calpain with the unextracted sedimented fraction in postmortem muscle; this μ-calpain was partly autolyzed.

INTRODUCTION

During meat aging extractable muscle m-calpain activity remains nearly constant, but the activity of extractable μ-calpain and calpastatin decreases. For this reason KOOHMARAIE et al. (1987) suggested that only μ-calpain, and not m-calpain is involved in postmortem tenderization. The rationale for this suggestion is that, once activated by Ca^{2+}, calpains undergo autolysis which eventually leads to loss of activity. However, results from a recent experiment seemed to contradict an important role for μ-calpain in postmortem tenderization. Intensive electrical stimulation (85 V, 14 Hz, 64 sec) resulted in about 80% reduction in extractable μ-calpain activity in bovine M. longissimus as soon as 1.5 h postmortem and about 75% reduction at 1 day postmortem, as compared to non-stimulated controls. Yet, only a slight (non-significant) difference in tenderness was noted at 1 day postmortem. Moreover, the rate and extent of tenderization in the stimulated muscles seemed not to be affected by the large reduction in extractable μ-calpain activity (GEESINK et al., 1994).

In one of the first papers on calpain in skeletal muscle it was suggested that part of the calpain is adsorbed to myofibrils (REVILLE et al., 1976). Preliminary results of our research on μ-calpain activity in human platelets indicates that μ-calpain, once autolyzed, becomes associated with the actin-, and membrane-cytoskeleton fractions, as defined by the method of FOX et al. (1992). These results prompted us to investigate whether a similar relocation of μ-calpain from the soluble to the insoluble protein fractions occurs in postmortem muscle.

MATERIALS AND METHODS

M. semimembranosus was obtained from a 18 month-old steer within 30 min after exsanguination at The University of Arizona Meat Science and Livestock Complex. The excised musle was trimmed free from external fat. The muscle was stored overnight at 15° C and kept at 4° C during subsequent storage. The muscle was sampled at 1 hour, 1 day, 2 days, and 3 days postmortem for partial purification of μ-, and m-calpain, and SDS-PAGE of myofibrillar proteins.

100 g of muscle was homogenized in 6 volumes of buffer (20 mM Tris/HCl, 5 mM EDTA, 0.1% ß-mercaptoethanol [MCE], 100 mg/l ovomucoid, 2.5 μM E-64, 2 mM phenylmethylsulfonyl fluoride, pH 8.0), using a Waring blender (2 x 20 sec. 18,000 rpm). The homogenate was centrifuged (15 min, 15000 x g), and the supernatant was filtered over glass wool. The pH was adjusted to 7.5 with solid Tris and brought to 0.5 M KCl with solid KCl. A small amount of the insoluble myofibrillar fraction was solubilized in SDS-PAGE sample buffer (0.05M Tris/HCl, 1% SDS, 0.01% Bromphenol blue, 30% glycerol, pH 6.8). Part of the muscle extract, equivalent to ca. 20 g of muscle, was loaded on a 25 x 1.6 cm hydrophopic interaction column (phenyl Sepharose. Pharmacia, 1 ml/min), that had previously been equilibrated with buffer A (20 mM Tris/HCl, 1 mM EDTA, 0.1% MCE, pH 7.5), containing 0.5 M KCl. The column was washed with buffer A, containing 0.5 M KCl, until the A_{280} reached the baseline, after which the bound proteins were eluted with buffer B (20 mM Tris/HCl, 1 mM EDTA, 0.1% EDTA, 10% ethyleneglycol, pH 7.5). Calpain-containing fractions were pooled and loaded on a 15 x 1.6 cm ion-exchange column (TSK DEAE-650 S, Supelco, 0.5 ml/min). μ-, and m-Calpain were separated with a linear gradient of 0 - 0.5 M KCl in buffer A. The active fractions were pooled (20 ml each), and concentrated to 1 ml with a Centriprep-30 (Amicon).

Proteolytic activity of the calpains was assayed at 25° C for 30 min using florescein isothiocyanate-labeled casein (FITC-casein) as a substrate as described by WOLFE et al. (1989).

SDS-PAGE was done according to WOLFE et al. (1989). After electrophoresis the proteins were electrophoretically transferred to a PVDF membrane (Millipore), using a semi-dry blotting apparatus (Millipore). The membranes containing the transferred proteins were incubated for 1 hour with monoclonal antibody D4F8 against the 80 kDa subunit of μ-calpain, monoclonal antibody 107 against the 80 kDa subunit of m-calpain, or monoclonal antibody 156 against the 28 kDa subunit of calpain. After washing, the membranes were incubated for one hour with a second antbody conjugated with alkaline phosphatase (American Qualex). Alkaline phosphatase development was used for the western blots.

RESULTS AND DISCUSSION

As expected, the extractable μ-calpain activity decreased during postmortem storage (Table 1). The low m-calpain activity at 2 days postmortem is likely due to reduced binding to the phenyl Sepharose column, which became partly clogged during loading of the sample.

A western blot against the 28 kDa subunit of the partly purified calpains is shown in Fig. 1. Although a decrease in the amount of the 28 kDa subunit of μ-calpain can be noted during postmortem storage, little or no intermediate autolysis products (as visible in the standards) or the final 18 kDa autolysis product can be detected. The amount of 28 kDa subunit of m-calpain reflects the measured amount of m-calpain activity (Table 1). Similarly, western blots against the 80 kDa subunit of m-calpain refelected the measured amount of m-calpain activity (data not shown).

A western blot against the 80 kDa subunit μ-calpain is presented in Fig. 2. As was observed for the 28 kDa subunit, a decrease

in 80 kDa subunit was observed during postmortem storage. Only a trace amount of the 78 and 76 kDa autolysis products could be detected in 3-days postmortem muscle.

A western blot against the 80 kDa subunit of μ-calpain in the sedimented muscle fraction is shown in Fig. 3. In the 1 hour postmortem sample, a small amount of native 80 kDa subunit could be detected. During postmortem storage an increasing amount of the autolyzed 78 and 76 kDa autolysis products could be detected in association with the sedimented muscle fraction.

These results indicate that μ-calpain becomes associated with the insoluble muscle fraction during postmortem storage, and that little of the partly autolyzed 78-76/18 kDa μ-calpain is extracted. Because the 78-76/18 kDa form of autolyzed μ-calpain retains full proteolytic activity, measurement of μ-calpain activity in postmortem muscle extracts underestimates the total amount of μ-calpain in postmortem muscle. These results may also explain the observations that a sharp decrease in extractable μ-calpain from electrically stimulated muscles did not significantly affect tenderization (GEESINK et al., 1994).

HATANAKA et al. (1984) observed that a small amount of μ-calpain in human erythrocytes was associated with the membrane cytoskeleton. Proteolytic activity of this membrane cytoskeleton-associated μ-calpain was not inhibited by calpastatin. Possibly, association of μ-calpain with the insoluble muscle fraction in postmortem muscle also abolishes the inhibiting action of calpastatin.

Table 1: μ-, and m-calpain activity at different times postmortem in bovine M. semimembranosus[a].

Time postmortem	μ-calpain	m-calpain
1 hour	131	151
1 day	46	104
2 days	27	28
3 days	18	91

[a]Calpain activity is expressed as fluorescent units (x 10^4) per gram of muscle.

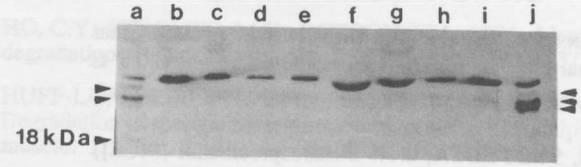

Fig. 1. Western blot using a monoclonal antibody against the 28 kDa subunit of calpain. Lanes a and j: standards of purified, partly autolyzed, bovine muscle μ-, and m-calpain, respectively. Lanes b-e: partly purified μ-calpain of M. semimembranosus at 1 hour, 1 day, 2 days, and 3 days postmortem, respectively. Lanes f-i: partly purified m-calpain of M. semimembranosus at 1 hour, 1 day, 2 days, and 3 days postmortem, respectively. Arrowheads indicate autolysis products of the 28 kDa subunit.

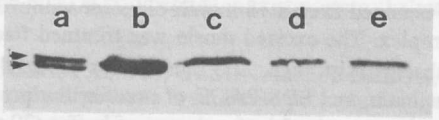

Fig. 2 Western blot using a monoclonal antibody against the 80 kDa subunit of μ-calpain. Lane a: standard of purified, partly autolyzed, bovine muscle μ-calpain. Lanes b-e: partly purified μ-calpain of M. semimembranosus at 1 hour, 1 day, 2 days, and 3 days postmortem, respectively. Arrowheads indicate the native 80 kDa subunit and the 76 kDa autolysis product.

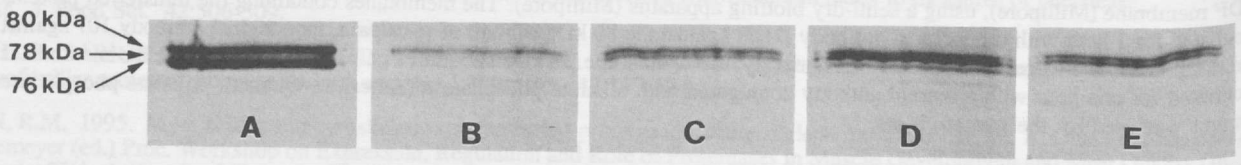

Fig. 3. Western blot using a monoclonal antibody against the 80 kDa subunit of μ-calpain. Lane a: standard of purified, partly autolyzed, bovine muscle μ-calpain. Lanes b-e: μ-calpain in the sedimented protein fraction of M. semimembranosus at 1 hour, 1 day, 2 days, and 3 days postmortem, repectively. Arrows indicate the native 80 kDa subunit, and the 78, and 76 kDa autolysis products.

CONCLUSIONS

Contrary to previous suggestions, μ-calpain is not autolytically or proteolytic degraded to inactive fragments during three days of postmortem storage. μ-Calpain in post-mortem muscles becomes associated with the unextracted sedimentable muscle fraction and little of the partly autolyzed 78-76/18 kDa μ-calpain is extracted. Therefore, measurements of extractable μ-calpain activity underestimate the proteolytic activity of this enzyme in postmortem muscle.

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