# EFFECT OF CALCIUM, μ-CALPAIN ON BEEF MYOFIBRILAR PROTEINS DEGRADATION UNDER POSTMORTEM CONDITIONS

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## Background

Tenderness for beef is the most important palatability characteristics, to a large extent, tenderness defines the value of beef (Boleman et al. 1997,Hill et al.2000).There are many factors affecting beef tenderness, such as the quality(cross-linkage) and quantity of connective tissue, fat deposit in intramuscular tissue (marbling ) etc. However, considerable experiments have demonstrated that it is the degradation of myofibrillar proteins contributing to tenderness during beef aging (Wulf et al. 1996,Wheeler et al.1994). Consequently, more research focus on myofibrillar /cytoskeleton proteins changes with beef postmortem storage, which is a disputed question until now. Koohmaraie et al. (1996) and Dransfield et al (1999) suggested that μ-Calpain plays a key role in myofibrillar proteins degradation. However, Takashi kouri et al. (1999) reported that calcium, instead of endogenous enzymes, alone could lead to myofibrillar protein degradation. Some other researchers (Calkins et al. 1988, Marie Lamare et al.2001) believe that lysosomal cathepsins and proteosome may also involve in beef postmortem tenderization.

## Objective

This study was performed for the purpose of understanding the degradation patterns of purified myofibrillar proteins when incubated with calcium and /or μ-Calpain under simulated beef postmortem conditions, and as a result, the mechanism of beef aging (tenderization).

## Methods

*Myofibrils preparation* Myofibrils were prepared from bovine longissimus dorsi within 1 h postmortem following the procedures of Huffer-lonergan et al. (1995). Purified myofibrils were stored in solution containing 50% glycerol, 100mM NaCl, 2mM $NaN_3$, at -30°C.

μ-Calpain, Calpastatin purification and activity assay μ-Calpain was purified according to Geensink et al. (1999) with minor modifications. Briefly, muscle was trimmed of visible fat and connective tissue within 1h postmortem and homogenized in three volumes of extraction buffer(100mM Tris-HCl, pH 8.3, 10mM EDTA, .05%[vol/vol] 2-mercaptoethanol [MCE],100mg/L ovomucoid, 2mM phenylmethylsulfonyl fluoride[PMSF], and 6mg/L leupeptin). After centrifugation, the supernatant was salted out between 0 and 45% ammonium sulfate saturation, and after being dialysized, μ-Calpain was purified using successive chromatography over DEAE-sephacel(Pharmacia Biotech AB), Phenyl-sepharose(Pharmacia Biotech AB), Butyl-sepharose(Pharmacia Biotech AB), DEAE-650S(Supelco), S-300(Pharmacia Biotech AB). The activity of resulted purified μ-Calpain (Fig. 1,2) and partly purified Calpastatin was assayed according to Koohmaraie et al. (1990). For preparation of partly purified Calpastatin, fractions containing Calpastatin activity were pooled after column DEAE-sephacel, and was heated for 15min at 90°C, after centrifugation, the supernatant was concentrated by polyethylene glycol 6000, then loaded onto S-300.

*Incubations* Myofibrils were washed three times in a mixed salt solution (MSS: 12mM NaCl, 12.6 mM $MgSO_4$ $7H_2O$, 70 mM $KH_2PO_4$, 3.4 mM NaOH, 64.2 mM KOH, 11.1 mM $H_2SO_4$, 132 mM Lactic acid, 100 mM Tris, 1% MCE, and 1 mMTris was added to adjusted pH to 5.8, 4°C) formulated on the basis of beef postrigor conditions. The protein concentration of the myofibril suspension was determined using the biuret assay. Altoghter, six treatments were designed, myofibril concentration in every incubation solution was adjusted to 5.8mg/ml by dilution using MSS, and the reaction volume was 2.5ml. Six treatments are as follows:

(1) myofibril (5.8mg/ml) (2) (1)+ Calcium (100mM) (3) (2)+ leupeptin (60mg/ml)
(4) (2) + leupeptin (120mg/ml) (5) (2)+ μ-Calpain(0.15U) (6) (5) +Calpastatin(0.6U)

*SDS-PAGE* After being incubated for 1d, 14d, 30d, at 5°C respectively, the reaction mixtures (400ul) were centrifuged at 6,000 $g_{max}$ for 5 min. the pellet was dissolved using 200ul SDS-PBS(2%SDS, 10Mm $Na_3PO_4$, pH 7.0), then 100ul sample buffer (3mM EDTA, 3% glycerol, 30mM Tris-HCl, 0.05% Bromophenol blue, pH8.0) and 20ul MCE were added. 100ul sample and 10ul MCE were incorporated into the supernatant. Sample were electrophoresied, after being heated for 20min at 50°C. Some gels were stained by commassie blue , then destained using destaining solution, others were used for western blotting.

*Western blotting* Proteins were electrophoretically transferred to PVDF membranes (Roche) for 1.5h at 4°C and 300mA in buffer containing 25mM Tris,195mM glycine, and 15% methanol using semidry transfer unit. To prevent nonspecific antibody binding, membranes were blocked with 5% nonfat dry milk in Tris buffed saline (pH7.4) containing .05%Tween-20 (TTBS) for 2.5h. Primary antibody used in these experiments included mouse anti-troponinT monoclonal antibody (JLT-12: 1:2000, Sigma). The secondary antibody was alkaline phosphatase conjugated anti-mouse IgG (Promega, 1:7000). Antibodies were diluted in blocking buffer, and incubations were for 1h at room temperature with gentle rocking. Membranes were washed three times with TTBS after each incubation. Antibody binding was visualized by exposure to BCIP/NBT(Roche).

## Results and Discussion

After incubation for 1d, 30KDa degradation polypeptide appeared in treatment 5, some degradation was also occurred for desmin(Fig.3,A). With the incubation time extended to 7d, 30KDa degradation products became more clear in treatment 5, meanwhile, 30KDa polypeptide also appeared (Fig.3B) in treatment 6. When incubated for 30d, more 30KDa degradation products appeared in treatments 5, 6 compared to the same treatment incubated for 3d, 14d respectively. At the same time desmin almost disappeared in these two treatments. However, there were not protein degradation differences in other treatments during incubation periods, and the appearance of 30 KDa degradation products. The results of western blotting of troponinT (Fig.4A,B) also indicated that μ-Calpain could degrade troponinT to 30 KDa or smaller molecules even in treatment with more Calpastatin activity. TropninT degradation fragments, especially, 30 KDa polypeptide is closely related to beef postmortem tenderness and can be used as a reliable tenderness predictor (Geensink et al. 2001, Basu et al. 2002). Desmin degradation is also positively related to beef tenderness. In this experiment, calcium alone could not degrade troponinT or desmin under simulated beef postmortem conditions, which suggested that ,to a large extent, μ-Calpain is responsible for beef postmortem tenderization.

## Conclusions

TroponinT and desmin, two important myofibrillar components, when incubated for 3d, 14d, 30d respectively with calcium and purified μ-Calpain under beef postmortem conditions, were degraded to smaller fragments. These fragments are closely related to beef tenderness. However, calcium alone could not degrade troponinT or desmin. Therefore, It is conclude that μ-Calpain play a key role in beef tenderization, and calcium affect beef aging indirectly by activating μ-Calpain.

## References

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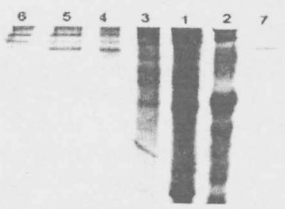


Fig1.Purification of μ-Calpain from bovine longissimus muscle.First to seven lanes: soluble extract, Ammonium sulfate fraction($P_{0-45}$), DEAE-sephacel, Phenyl-sepharose, Butyl-sepharose, DEAE-TSK, Sepharycel-300, proteins were resolved on 12.5% gel.

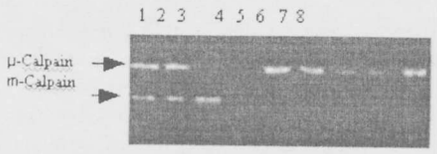


Fig2. Casein zymography of calpains at different purification steps.
1.soluble extract; 2.Ammonium sulfate fraction ($P_{0-45}$); 3,4. DEAE-Sephacel; 5.Phenyl-sepharose; 6.Butyl-sepharose;7.DEAE-TSK;8.S-300

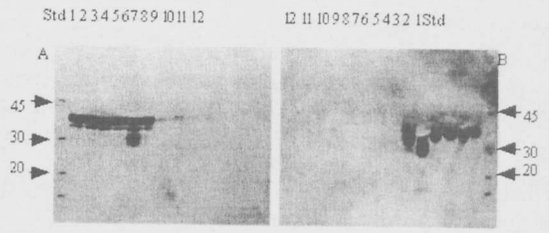


Fig.4. Western blotting (12.5%) of tropornin-T degradation products.
A, B indicate two different incubation periods (1, 14, days).1, 2, 3, 4, 5 and 6 represent samples from pellet fraction; 7, 8, 9, 10, 11 and 12 from supernatant fraction respectively.

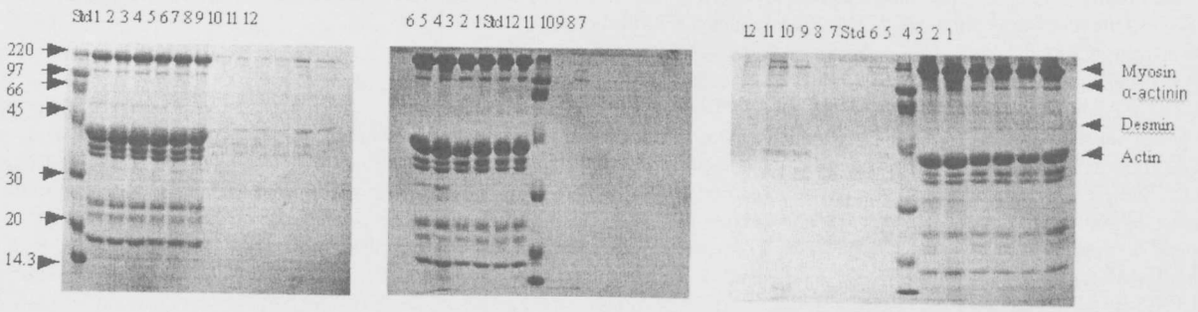


Fig.3. SDS-PAGE (12.5%) from six reaction mixtures, A,B and C indicate three incubation periods (1, 14, 30 days respectively).1, 2, 3, 4, 5 and 6 represent samples from pellet fraction, 7, 8, 9, 10, 11 and 12 supernatant fraction respectively. Std is the molecular standard lane

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