EFFECTS OF EXOGENOUS PROTEASE EFFECTORS ON CALPAINS ACTIVITY AND ULTRASTRUCTURAL CHANGES OF BEEF

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Background

Beef tenderness increases gradually during postmortem aging, and as a result, the improved palatability. This is an established phenomenon which has long been discovered, however, to date, which factors contribute to this process and how the tenderization is initiated is an unresolved problem.

Koohmaraie and others(Koohmaraie et al.1996, Dransfield 1999) suggested that postmortem meat tenderization is primarily the result of calpain(especially μ -calpain)-mediated degradation of key myofibrillar and cytoskeletal proteins which can cause weakening of the muscle structure, and therefore, meat becomes tender. On the contrary, Takahashi et al. (1999) supposed that with the postmortem time extended, the concentration of calcium ion in muscle sarcoplasm increased, and the calcium alone could lead to the degradation of myofibrillar proteins and the improved meat tenderness, and that calpain was considered to be inactive due to the low concentration of sarcoplasmic calcium ions, and therefore, has little contribution to the tenderization of meat throughout postmortem aging. Consequently, in order to control and regulate meat tenderization, it is indispensable to elucidate the mechanism of meat aging.

Objectives

The purpose of this study was to, through injection treatments, investigate the role of calcium or calpains in beef aging *in situ*.

Materials and methods

Sampling procedures and treatment of muscle samples

Three 2.5 years old Chinese yellow cattle (Luxi×Limusin with liveweight 500±20kg) were slaughtered according to standard slaughtering practices. After being chilled for 20h, one strip loins were sampled per cattle, and was divided into 21 steaks. After being trimmed of all external fat and connective tissue, the steaks(25±3g) were randomly assigned to seven sample groups, which were injected (10%raw weight) distilled water(control),200mM$CaCl_2$, 200mMEGTA, 200mM$ZnCl_2$, 0.2mg/ml Leupeptin ,0.2mg/ml Leupeptin plus 1%TritonX-100 and 1%TritonX-100 respectively. Then the samples were vacuum packaged individually, and stored at 4℃ for 3, 8, 16 days respectively.

Casein Zymography

Casein Zymography was based on the protocol described by Veiseth et al.(2001) with minor modifications. At each storage time, 300mg of muscle were homogenized in 3vol of extraction buffer (100mMTris,10mMEDTA,0.05%2-mercaptoethanol[MCE],pH8.3) using hand operated homogenizer.The homogenate was centrifuged at 10,000×g_{max} for 20min, and the volume of the supernate was measured and used for casein zymography. Hammersten Casein(0.2%, wt/vol) was incorporaed in 12% separating gels(80:1 ratio of acrylamide to bisacrylamide, 375mM Tris-HCl, pH8.8), and 4% stacking gels(80:1, 125mMTris-HCl, pH6.8) without Hammersten Casein was used. TEMED and ammonium persulfate were used to catalyse the polymerisation. Sample buffer(150mM Tris-HCl, pH6.8, 20% glycerol, 0.8%MCE, 0.02% bromophenol blue) was added to supernate (4:1 ratio of buffer to supernate). The gels(1mm) were prerun at 100V for 15 min, 4℃, with a running buffer containing 25mMTris-HCl, 0.05%MCE,192mM glycine, and 1mMEDTA (pH8.3) before samples were loaded into the wells. The gels were run at 100V for 15h, 4℃, removed, and incubated at room temperature (RT) in 50mMTris-HCl, 0.05%MCE and 4mM $CaCl_2$(pH7.5) with slow shaking for 1h, then followed by 16h incubation in the same buffer at RT, before staining for 1h with Coomassie blue and destaining with 20% methanol and 7%acetic acid.

Muscle ultrastructure

At 3, 8, and 16 days storage periods, samples were cut parallel to muscle fibre and fixed by immersion in cold 2.5% glutaraldehyde in phosphate buffer (pH7.3), postfixed in 1% osmium. dehydrated in ethanol, embedded in Epon812 resin. For all samples, thin sections of the same thickness were cut parallel to the

fibres. Sections were stained with uranyl acetate and lead citrate, and then examined using a JEM-100CX-Ⅱ transmission electron microscope(TEM).

Results and discussion

Effects of injection treatment on calpain activity

Results presented in Figure1 indicate that the μ-calpain activity treated by distilled water, EGTA and TritionX-100 respectively declined quickly with aging time and after 16 days storage, μ-calpain activity could not be detected at all by Casein zymography. In contrast, μ-calpain activity in samples injected with $ZnCl_2$, Leupeptin, and Leupeptin plus TritionX-100 respectively can still be detected up to 16 days postmortem. As compared to μ-calpain activity , m-calpain activity did not changed dramatically at a 16 days storage period in all treatments except for $CaCl_2$ injected group in which both μ-calpain and m-calpain activity almost lost completely even after 3 days storage. Two or more band showing activity also appeared in figure 1. Calpain system occurring in animal skeletal muscle cells has three major components ie. μ-calpain, m-calapin and their specific inhibitor calpastatin. A unique property of the calpains is that, when exposed to sufficient calcium, they undergo autolysis, which initially reduces the 80kDa subunit of calpain to 78 or 76kDa, and the 28kDa subunit to 18kDa (Geensink et al.2000). Further autolysis leads to more extensive degradation of large subnunit and loss of proteolytic activity. Znic chloride, Leupeptin are exogenous inhibitor of calpains, the activity of μ-calpain in muscles incorporated into these two components, therefore, declined more slower compared to other treated groups. Calpains quickly lost activity in $CaCl_2$ injected group, which demonstrated that sufficient calcium can activate calpains and then calpains themselves lose activity through autolysis. Two or more active bands of μ-calpain supported the calpain autolysis theory. Except for $CaCl_2$ treated samples, the activity of m-calpain did not change greatly, which indicated that under the condition of this experiment, m-calpain kept inactivity due to low level of calcium, and hence, is not a potential contributor to beef tenderization.

Effects of injection treatment on beef ultrastructure

These experiments conducted demonstrated that injected treatments had great influence on beef ultrastructural changes (Fig.2). Myofibrils kept intact at d3 postmortem (Fig.2 A). At d16, some breaks in myofibrils appeared in control groups (Fig.2 B). While in Leupeptin treated samples, even after 16 days of storage, there was not any significant changes occurred for myofibrils (Fig.2 C). By contrast, myofibrillar breaks was readily perceived at d3 in $CaCl_2$ treated group (Fig.2 D). It has been well documented that muscle fiber changes were mainly caused by the degradation of key myofibrillar proteins, especially, titin, nebulin desmin etc. (Koohmaeaie 1996). When calpains were inhibited by Leupeptin, muscle ultrastructural changes were also inhibited, while, when calpains were activated by calcium, muscle ultrastructural changes quickly happened, which is consistent with calpain hypothesis. In agreement with Taylor et al.(1995), another change that occured in muscle during postmortem tenderization was the fracture in the I band adjacent to the Z line(Fig.2 E), not in the middle of Z line as suggested by other reporters.

Conclusions

Calpain, especially μ-calpain is the most important factor responsible for meat tenderization, while calcium may has indirect influence on meat aging by activating calpains.

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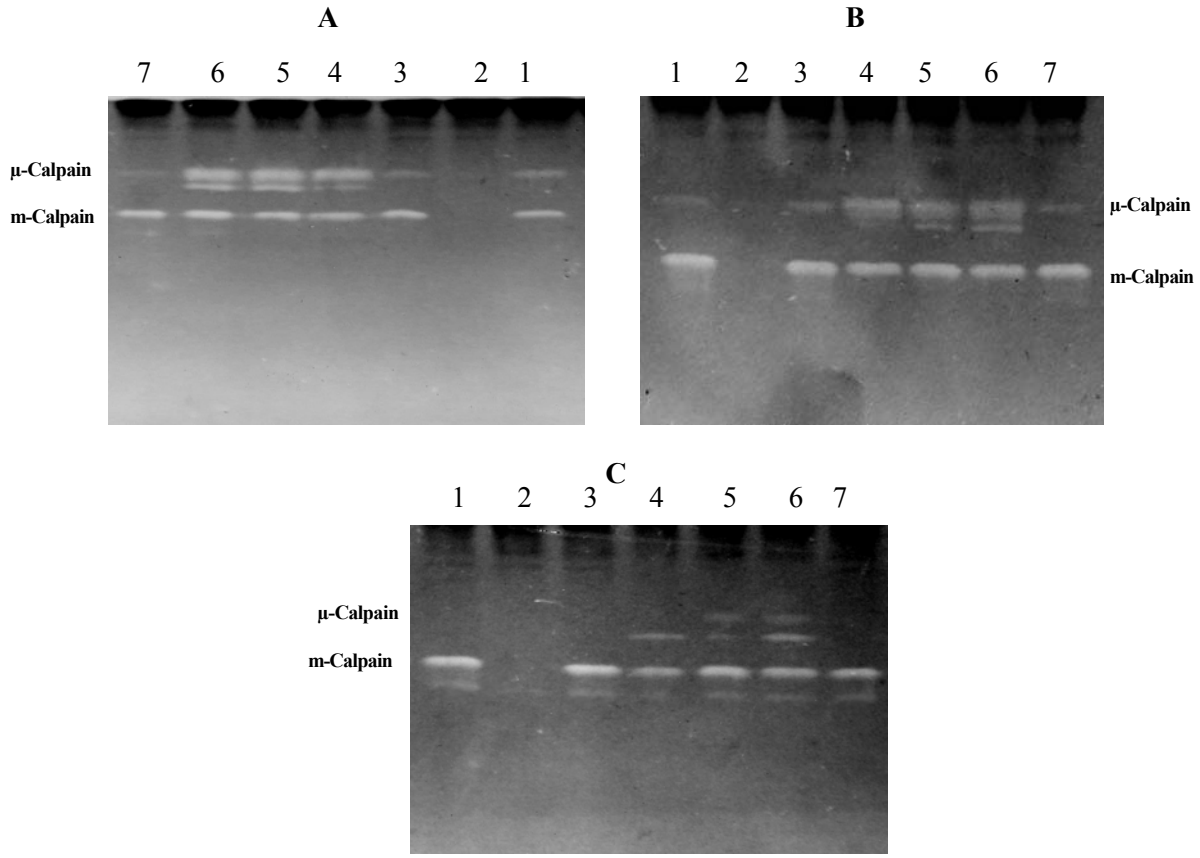

Fig.1 Casein zymography profiles(A, B, C) of beef calpains at 3d, 8d, 16d postmortem respectively. Lane 1 distilled water. Lane 2 calcium chloride. Lane 3 EGTA. Lane 4 Znic chloride. Lane 5 Leupeptin. Lane 6 Leupeptin plus Triton X-100. Lane 7 Triton X-100.

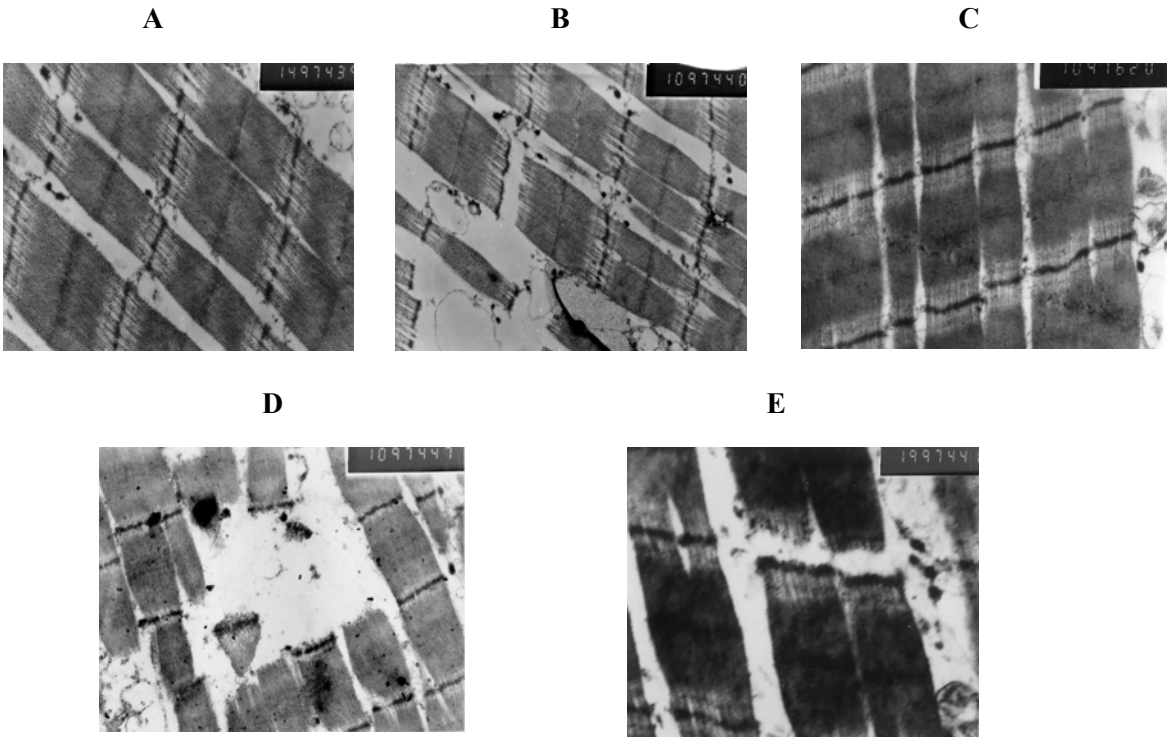

Fig. 2 Micrographs of ultrastructure of different treated beef .
A control sample aged for three days B control sample aged for sixteen days.
C Leupeptin treated sample aged for sixteen days D calcium treated sample aged for three days.
E. location of myofibril breaks.

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