EFFECT OF CALPAIN EFFECTORS ON TENDERNESS, CALPAIN ACTIVITY AND ULTRASTRUCTURE OF BEEF FROM CHINESE YELLOW CATTLE

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***Abstract*—the objective of this study was to investigate the biochemical factors affecting beef postmortem tenderization. After slaughter and chilled for 20 h at 4 ℃, beef strip loins were obtained and injected with distilled water, calcium chloride ($CaCl_2$), EGTA, zinc chloride ($ZnCl_2$), leupeptin or Triton-X-100, then vacuum packaged, and stored at 4 ℃ for 3, 8 and 16 d, respectively. Beef tenderness, calpain activity and ultrastructure were detected. Results indicated that calcium injection increased rate of beef tenderization ($P < 0.01$); μ-calpain activity was extended greatly in $ZnCl_2$ and leupeptin samples, but μ/m-calpain activity disappeared quickly in calcium samples. Compared to control, ultrastructural disruption occurred more quickly in calcium samples, whereas there was little structural change in leupeptin samples. These results suggest that calpains, especially μ-calpain, play a key role in beef aging and calcium accelerates beef tenderization possibly through activating μ and m-calpain or other uncharacterized cysteine endopeptidases.**

***Index Terms*—beef, Chinese yellow cattle, calpain effectors, tenderization, ultrastructure**

I. INTRODUCTION

It has been generally accepted that myofibrillar and cytoskeletal proteins degradation by endogenous protease are the major cause leading to the weakening of the structures of myofibrils and as a result, the improved meat tenderness. Calpain has been considered as a important contributor to postmortem tenderization, but there are still some disputing about the protease. Kanawa, Ji and Takahashi (2002) reported that μ-calpain was inactive throughout post slaughter aging. Camou, Marchello, Thompson, Mares and Goll (2007) also found that there was little μ-calpain activity after 48 h of postmortem storage. They all believed that μ-calpain was not responsible for postmortem proteolysis, at least beyond 2 to 3 days postmortem. In order to advantageously exploit and regulate meat aging, the mechanism underlying meat tenderization should be elucidated, especially the role of calpains and calcium. The objective of this study was to investigate *in situ* the role of calpains or calcium in beef from Chinese yellow cattle postmortem aging through injection of calpain effectors $CaCl_2$, EGTA, $ZnCl_2$, Triton-X-100 and leupeptin, thus providing theoretical guidance for improvement of local beef quality.

II. MATERIALS AND METHODS

Six 3.5 years old Chinese yellow cattle (Luxi bulls with live weight 500 ± 20 kg) were slaughtered humanely according to the *Requirements of Islamic Slaughtering*. After being chilled for 24 h at 4 ℃, strip loins were obtained, and each was cut into three steaks with 2.54 cm thick and 21 small cubes (35 ± 3g). the cubes were injected (10% vol/wt) with distilled water (control), 200 mM $CaCl_2$, 200 mM EGTA, 200 mM $ZnCl_2$, 0.2 mg/ml leupeptin, 0.2 mg/ml leupeptin plus 1% Triton-X-100 or 1% Triton-X-100. After a 10 min equilibration time, the samples were vacuum packaged individually, stored at 4 ℃ for 3, 8, 16 days, respectively, and subjected to the assay of Warner-Bratzler shear force (WBSF), calpain activity and ultrastructure of myofibrils.

III. RESULTS AND DISCUSSION

Effect of injection treatment on beef tenderness: Injection treatment had significant influence on beef tenderization (Fig.1). At 3 days postmortem the WBSF of $CaCl_2$ injected samples decreased to 3.5 kg which was similar to that of control aged for 16 days ($P > 0.05$). During the time interval between 3 and 16 days postmortem, the decline of WBSF of $CaCl_2$ injected samples was much less than that of control. Hence, it is apparent that $CaCl_2$ accelerates beef tenderization. Leupeptin injection, however, inhibited beef tenderization dramatically ($P < 0.01$). During 18 days of aging, the WBSF of the leupeptin treated samples was still as high as 5.1 kg, just 0.3 kg decline. Similarly, it was

reported that injection of leupeptin blocked beef tenderization completely (Uytterhaegen, Claeys & Demeyer, 1994). Hopkins and Thompson (2001) also found that calpain inhibitor can prevent tenderization when injected into pre-rigor lamb. It has been confirmed that leupeptin is a specific inhibitor for cysteine proteinase, therefore, the role of cysteine proteinases including calpain is necessary in beef postmortem tenderization.

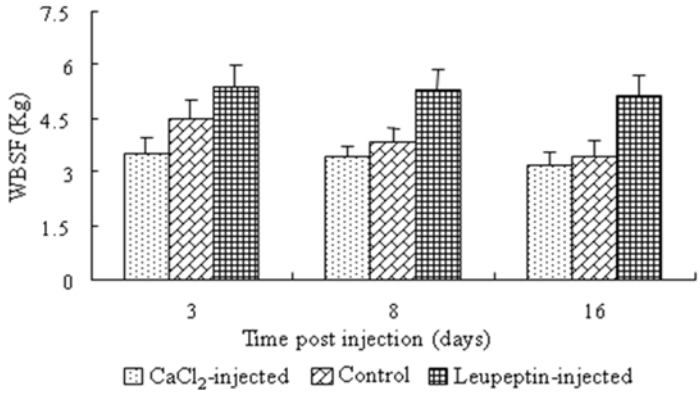

Fig.1 Changes in Warner-Bratzler Shear Force (WBSF) of beef strip loins injected with different calpain effectors ($CaCl_2$ and leupeptin) after storage of 3, 8 and 16 d at 4 ℃. Vertical bars represent standard deviation of the mean of 6 replicates.

Effect of injection treatment on calpain activity: Results presented in Fig.2 showed that the μ-calpain activity following treatment with distilled water, EGTA and Trition-X-100, respectively declined gradually with aging time. At 3 d and 16 d postmortem, μ-calpain activity could not be detected by Casein zymography in $CaCl_2$ treated and control samples[Fig. 2 (a) and (c)], whereas, μ-calpain activity in samples injected with $ZnCl_2$, leupeptin, and leupeptin plus Trition-X-100 was still readily detectable up to 16 d postmortem [Fig. 2 (c)]. In contrast, m-calpain activity did not changed dramatically over a 16 days of storage period in all treatments (Fig. 2) except for $CaCl_2$ injected group in which both μ-calpain and m-calpain activities almost lost completely even within 3 days of storage [Fig. 2(a)].

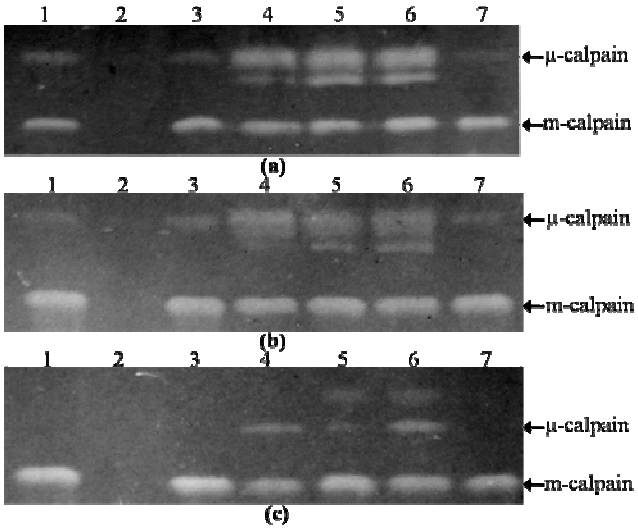

Fig.2 Representative casein zymography gel depicting μ-calpain and m-calpain activity of beef samples injected with different calpain effectors. (a), (b), (c) indicate storage of 1, 7 and 14 d at 4 ℃, respectively. Lanes 1-7 represent samples treated with distilled water, $CaCl_2$, EGTA, $ZnCl_2$, leupeptin, leupeptin plus Triton-X-100, and Triton-X-100, respectively

In the present experiments, except for the $CaCl_2$ treated group, multiple bands indicating μ-calpain activity appeared in all casein zymography profiles (Fig. 2), which demonstrated that the autolysis of μ-calpain also occurred in beef postmortem. However, the activity of μ-calpain declined with extended time which is possibly due to the autolyzed μ-calpain becoming unstable and loss of activity at high ionic concentrations accumulated in beef postmortem (Veiseth, Shackelford, Wheeler & Koohmaraie, 2004). Although to date, there is still disagreement on the specific concentrations of calcium, it is generally accepted that a certain concentrations of calcium is indispensable for activating native or autolysed calpains. Results showed in this study that although after a given time postmortem μ-calpain is activated, calcium concentration in beef itself is not enough to activate m-calpain and thus m-calpain does not play a major role in beef tenderization, However $CaCl_2$ injection is an effective way to activate m-calpain, which is confirmed by its quick loss of activity detected by casein zymography (Fig. 2).

Leupeptin, $ZnCl_2$ 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 (Fig. 2). Although leupeptin can also inhibits other cysteine proteinases, such as cathepsin B, cathepsin L, and among others, many experiments evidenced that calpains, especially μ-calpain, are the determinant endogenous biological factors responsible for meat aging (Koohmaraie 1996).

In samples injected with the detergent Trition-X-100, which destroy membrane system such as cytoplasm reticulum and mitochondria containing rich calcium, thus enhancing the release of calcium into cytosol, then the calpain is activated, autolyzed and lost activity more quickly. It was also documented that meat injected with Trition-X-100

accelerated meat aging and resulted in significantly lower shear force values.

Effects of injection treatment on beef ultrastructure: Our experiments demonstrated that injection treatments had a great influence on ultra-structural changes in beef (Fig.3). In control groups myofibrils remained intact at 3 d postmortem [Fig.3 (a)], at 16 d, some breaks in myofibrils appeared [Fig.3 (b)], while in leupeptin treated samples, even after 16 days of refrigerated storage, there was not any significant changes occurred for myofibrils [Fig.3 (c)]. By contrast, myofibrillar breaks were readily perceived at 3 d in $CaCl_2$ treated group [Fig.3 (d)].

Consequently, when calpains were inhibited by leupeptin, muscle ultra-structural changes were delayed markedly, while, when calpains were activated by sufficient calcium, muscle ultra-structural changes were quickly initiated, from which it can be concluded that the postmortem aging is very likely mediated by calpains. Meanwhile, we also found another change that occurred in muscle during postmortem tenderization was the fracture in the I band adjacent to the Z-line [Fig.3 (e)], not in the middle of Z-line as suggested by Ahn, Shimada and Takahashi (2003), who believed that calcium alone can result in the phospholipid liberation from Z-line and leading to the weakening of Z-line and the disruption of myofibrils at Z-line level. The latter reports generally based on in vitro experiments and hypothesis, and thus more experiments may be performed before drawing a conclusion.

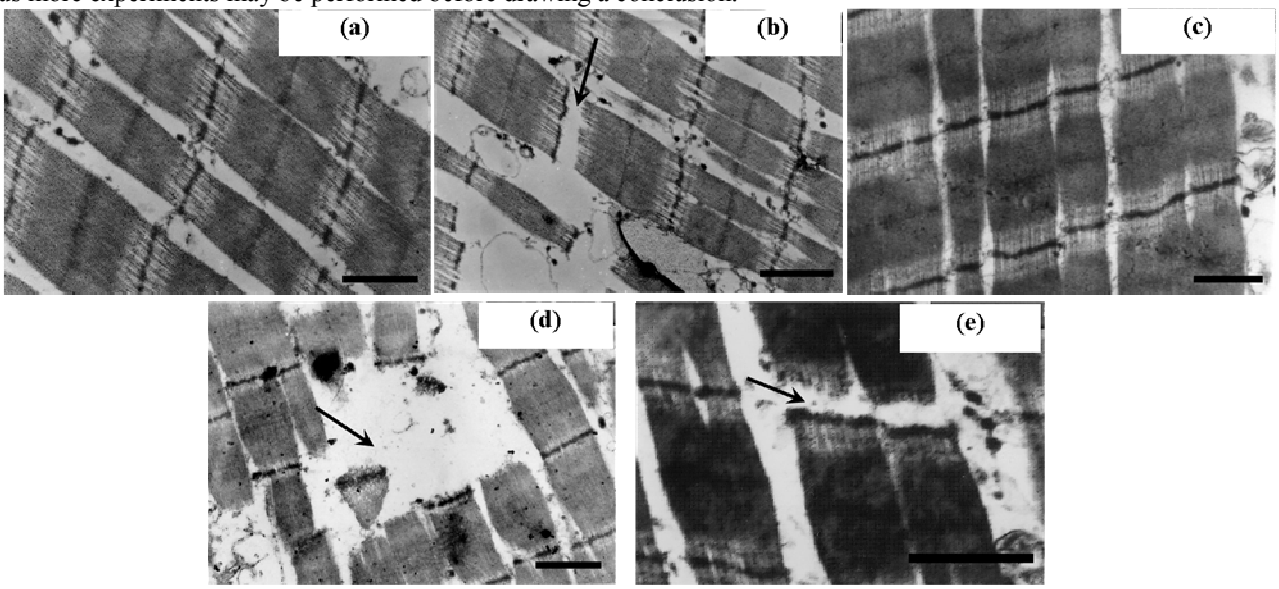

Fig.3 Representative transmission electron micrographs depicting major changes in ultra-structure of beef longissimus postmortem: (a) control sample aged for 3 days; (b) control sample aged for 16 days (arrow showing the breaks); (c) leupeptin injected sample aged for 16 days; (d) $CaCl_2$ injected sample aged for 3 days (arrow showing the breaks); (e) showing the location of myofibril breaks adjacent to Z-line (arrow). Scale bar: 1.0 μm.

IV. CONCLUSION

The results presented here strongly suggest that endogenous cysteine endopeptidases, most likely μ-calpain, are the important biochemical factors responsible for meat tenderization from Chinese yellow cattle. Although increasing the concentration of free calcium in muscle fibers is an effective way of accelerating meat aging, calcium alone is not responsible directly for a major role in meat tenderization, but is most likely achieved by activating μ-calpain and/or m-calpain to exert its influence on meat aging.

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