Effects of the inhibitors of calpain MDL-28170 and Calpeptin on caspase-3 activity of chicken during postmortem ageing

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Abstract— **Calpain has been considered to be the most important protease involved in tenderization during the conversion of muscle into meat. However, recent evidence suggests the possible involvement of the key apoptosis protease, caspase, on the underlying mechanism of postmortem tenderization. In this study, we used inhibitors of calpain to treat chicken muscle immediately after slaughter, and followed the changes in caspase-3 activities together with the energy changing during 5 days of ageing. Addition of calpain inhibitors to the system resulted in significantly higher caspase-3 activities ($p<0.01$) during storage by using spectrofluorometer. Western blot analysis of α-spectrin cleavage of the 120 kDa peptide (SBDP 120), showed that the addition of calpain inhibitors resulted in the formation of higher amounts of the active form of capsase-3 compared with the control ($p<0.01$). The findings of this investigation show that calpain prevented the activation of caspase-3 during postmortem ageing. We therefore suggest that there is a relationship between caspase-3 and calpain which contributes to the conversion of muscle tissue into meat..**

Keywords— **caspase-3; ageing; apoptosis**

I. INTRODUCTION

These The tenderizing of meat during the ageing process is highly complex and although there still some controversy regarding the actual mechanism, it is generally accepted that major components affected by proteolysis are the key myofibrillar proteins [1]. Several studies suggest that some of the changes occurring in mammalian muscle ultrastructure during ageing are associated with the action of calpains; moreover, there is much evidence that the calpain system plays a primary role in proteolysis [2]. Apoptosis is a common physiological event that occurs in proliferating and regenerating tissues. Caspase-3, a member of the interleukin-1β-converting enzyme family of cysteine proteases, is a trigger for the execution phase of apoptosis [3, 4]. Furthermore, there is growing evidence indicating a significant cross-talk and functional connections between calpains and caspases in the regulation of apoptosis [5, 6]. Since the calpain system has been considered most important the initial tenderizing process of meat, the recent implication of caspase-3 as playing an additional role has prompted questions regarding the actual relationships or interactions between these two proteolytic enzymes. Based on the available evidence, calpains have the potential to both positively and negatively modulate the caspase cascade during apoptosis, thus determining the relative roles of the two proteolytic systems in meat ageing. We have designed this investigation to examine interactions between the calpain and caspase protease systems in postmortem chicken meat by using the inhibitors MDL-28170 and calpeptin to prevent function of calpain. The objective of this study was an attempt to demonstrate the possibility of cross-talk between the two proteolytic systems, calpain and caspase-3. Moreover, we provide further evidence regarding the role of caspase in the tenderization of chicken meat.

II. RESULTS

A. Caspase-3 activity

The conversion of the substrate Ac-DEVD-AFC into free AFC was used as a measure of caspase-3 activity in the presence and absence of a calpain inhibitor. Compared with the initial caspase-3 activity on day 0 there was a significant increase in the Control and treatment groups by day 1 (Table 1). By day 3 caspase-3 activities had commenced to fall and by day 5 of ageing the activities of all samples had reduced by about 50 % compared with those measured on day 1. The caspase-3 activities in the presence of MDL-28170 or calpeptin were significantly higher ($p<0.01$) on each of the 5 days postmortem meat ageing period as compared to control (Table 1).

Table 1. Caspase-3 activities of chicken meat in the absence (Control) and presence of calpain and calpeptin the inhibitor, MDL-28170 during storage at 4 °C for 0, 1, 3 or 5 days. . Activity

was based on the conversion of the substrate Ac-DEVD-AFC to free AFC.

Days of storage	Caspase-3 activity		
	Control	MDL28170	Calpeptin
0	48.34±3.32	-	-
1	61.76±1.99[a]	177.26±4.33[b]	140.62±2.40[b]
3	34.41±3.82[a]	51.33±3.66[b]	67.56±2.32[b]
5	20.53±0.75[a]	82.32±2.16[b]	85.80±4.50[b]

Note: Values are expressed as absorbance (arbitrary units) per mg protein. Each value represents the mean ± SD, n=4

[a,b,] : Means within a row with different superscripts are significantly different ($p<0.01$).

B. Determination of the relative activities of caspase-3, and its degradation of α-spectrin

Caspase-3 is synthesized as a precursor molecule, which is approximately 32 kDa in size and is activated by intra-chain proteolytic cleavage during its conversion to two subunits (Ito, Oh-hashi, Kiuchi & Hirata, 2006). Using immunoblotting, we have demonstrated that the pro-form of caspase-3 was decreased during postmortem storage. Furthermore, several bands of 17~20 kDa corresponding to caspase-3 were detected in our samples (Figure 1). Where the calpain inhibitors were present, it can be seen that both MDL-28170 and Calpeptin resulted in significantly lower amounts of the pro-form of caspase-3 compared with the control relative to 0 day ($p<0.01$, Figure 1).

Caspase-3 mediated cleavage of α-spectrin generates 120 kDa degradation products (SBDP120), but α-spectrin can also be cleaved by calpains producing a 150 kDa peptide (SBDP150). In this study western blots probed with anti-α-spectrin detected immunopositive bands of intact 240 kDa, α-spectrin, and cleavage products, 150 and 120 kDa (Fig. 2(A)). Treatment of muscle with MDL-28170 or Calpeptin both significantly ($p<0.01$) reduced the degradation of α-spectrin, resulting in less of the 150 kDa fragment, These differences, compared with the controls, were significant for each of the storage days when using the 150 kDa of 0 day as a marker for each of the western-blot images ($p<0.01$) (Figure 2 (B)). However, the calpain inhibitors increased the 120 kDa fragment (Figure 2(A)), showing very high intensity values of the SBDP120 in the treated groups during 5 days of postmortem ageing (Figure 2(C)).

These results show that the inhibitors of calpain, MDL-28170 and Calpeptin both enhance the level of caspase-3 cleavage. The two inhibitors have similar enhancing effects on the activity of caspase-3 during the tenderization of chicken.

Fig. 1. Cleavage of caspase-3 in chicken breast muscle preparations in the absence (CON) and in the presence of 100 μM MDL-28170 (MDL) or 100 μM calpeptin (CAL) when stored at 4 °C for 0, 1, 3 or 5 days as shown by western blots with anti-caspase-3 (A); together with the relative values of caspase-3 during 5 days of storage at 4 °C (B). Fourty μg of protein from each sample was loaded onto each lane (A). Relative values were calculated as the blot intensity of the full-length caspase-3 at 0 day (B). Mean ± SD (n=4). **: $p<0.01$ vs. control. GAPDH showed that equal amounts of protein were applied to each lane.

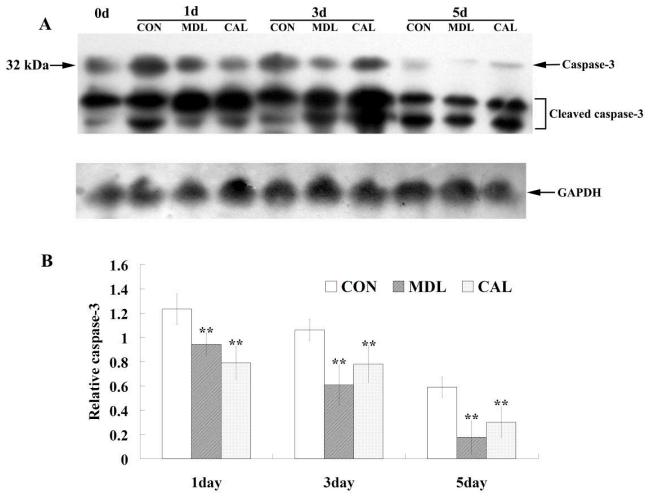
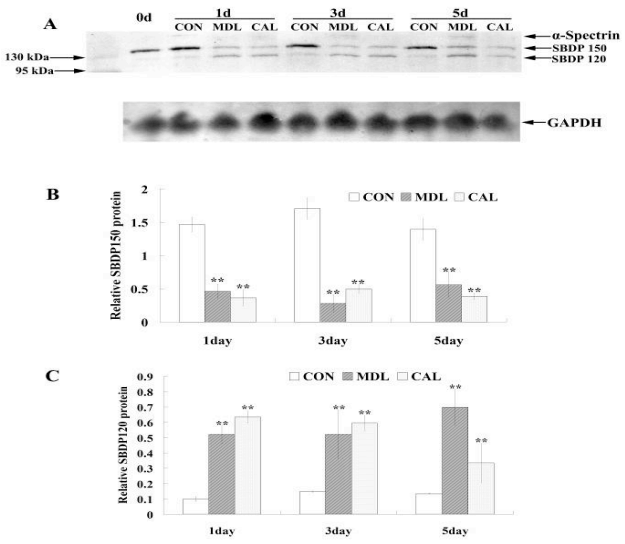


Fig. 2. Cleavage of α-spectrin of chicken breast muscle preparations in the absence (CON) and in the presence, of 100 μM MDL28170 (MDL) or 100 μM calpeptin (CAL) when stored at 4 °C for 0, 1, 3 or 5 days as shown by western blots with anti-α-spectrin (A); together with the relative values of 150 kDa (B) and 120 kDa(C) cleavage products of α-spectrin (SBDP150 & SBDP120) during 5 days of storage at 4 °C. Fourty μg of protein from each sample was loaded onto each lane (A). Relative values were calculated as the blot intensity of 150 kDa fragment of α-spectrin at 0 day (D). Mean ± SD (n=4). **: $p<0.01$ vs. control. GAPDH showed that equal amounts of protein were applied to each lane.



III. RESULTS

Our findings demonstrate that calpain down-regulates caspase-3 activity, viz. the consequence of calpain inhibition in this model is up-regulation of the caspase cascade based on increased caspase-3 activity measured by in vitro fluorometric assay; increased proteolytic processing of procaspase-3 to its active form; and increased cleavage of substrate α-spectrin to its signature 120 kDa caspase-derived breakdown product. Interpretation of these studies is further complicated by the potential for cross-talk between the calpain and caspase protease systems by commonly used caspase inhibitors.

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