Influence of Ultrasound and Proteolytic Enzyme Inhibitors on Muscle Degradation,Tenderness, and Cooking Loss of Hens During Aging

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Abstract

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The potential contribution of mechanical disruption by ultrasonics and endogenous proteolytic enzymes on the tenderisation of hen muscle were investigated. The importance of endogenous enzymes was evaluated using various specific inhibitors. Freshly obtained breast muscles of culled hens, from the 6 groups investigated were treated with different proteolytic enzyme inhibitors and/or ultrasonics, group was treated with different methods, and then stored at 4°C for 0, 1, 3, and 7 days. Shear force decreased by 1.19 kg, and shear force and cooking loss were reduced by 0.69 kg and 4.27%, respectively, in the incorporated group treatment. The calpastatin activity was affected by all treatments except in the Z-DEVD-fmk-treated group, caspase-3 activity decreased in Z-DEVD-fmk-treated group. Therefore, the results suggest that ultrasonics and endogenous proteases contributed to muscle degradation, thereby improving hen meat tenderness and decreasing the cooking loss. Thus muscle degradation, tenderness, and water-retaining properties of hens were improved by a combination of ultrasound and exogenous proteolytic enzyme inhibitors.

Keywords: ultrasonic; protease inhibitor; incorporating treatment; myofibrillar protein; Caspase-3/7; Z-DEVD-fmk

The quality of meat is determined by its visual appearance, organoleptic properties, chemical composition, and its physical attributes such as texture (Papadima *et al*. 1999; Probola & Zander 2007). The desirable attributes of meat palatability are mainly determined by tenderness, flavour, and juiciness. Tenderness is affected by the composition, organisation of myofibrillar structure, and by muscle integrity. It has been well established that post mortem degradation of specific myofibrillar proteins is closely linked to the structural changes which result in increased tenderness of meat during aging (Taylor *et al*. 1995; Koohmaraie 1996; Geesink *et al*. 2001; Huang *et al*. 2009). Four major proteolytic enzyme systems have been identified in skeletal muscles causing the degradation of proteins into amino acids or small peptides. These are lysosomal and proteasomal systems which result in more general proteolysis, while the calpain and caspase systems (Huang *et al*. 2009; Costelli *et al*. 2005) result in a more limited and specific proteolysis and play an important role during the early *post mortem* stages.

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Tenderisation during aging has been mainly ascribed to the proteolytic decomposition of myofibrillar proteins, titin, nebulin, troponin-T, desmin, and filamin (Huff-Lonergan *et al*. 1996), while the most prominent breakdown product is derived from troponin-T (Bowker *et al*. 2008; Chen *et al*. 2011). The presence of a 30 kDa fragment of troponin-T has been found in aged beef (Ho *et al*. 1996), chicken (Mikami *et al*. 1987), pork (Lametsch *et al*. 2002), and lamb (Kuber *et al*. 2003).

Caspases, a family of cysteine proteases, have been shown to be important during *post mortem* conditioning of meat. Kemp *et al*. (2006) investigated the relevant expression of various caspase types in different porcine skeletal muscles and reported the direct involvement of the caspase family in meat tenderisation. Moreover, Huang *et al*. (2009) made use of DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-CHO), a selective inhibitor of caspase-3, and showed that it dramatically reduced the degradation of the skeletal proteins, such as nebulin, titin, troponin-T, and desmin, while the activity of calpain was not affected. Chen *et al*. (2011) used apoptosis inducers, camptothecin and etoposide as well as Ca^{2+} and found that myofibrillar decomposition and proteolysis were both accelerated by the use of each of the three apoptosis inducers. As a result of the high activity of caspase-3 during the early p*ost mortem* conditioning period, it is likely that caspase-3 contributes to the degradation of skeletal muscle proteins (Chen *et al*. 2011). Therefore, it has been suggested that the degradation of skeletal muscle proteins should not be solely ascribed to the calpain system, and that the caspase family may play an important role in post mortem tenderisation (Huang *et al*. 2009; Chen *et al*. 2011).

Ultrasound treatment has been widely used experimentally for the tenderisation of meat (Stadnik *et al*. 2008; Siró *et al*. 2009; Farid *et al*. 2011). It is used for its ability to cause cell membrane destruction resulting from cavitation. The reasons for its effectiveness in improving meat tenderness have been ascribed to physical disruption/softening of skeletal muscle structure, or indirectly, to the activation of Ca-dependent proteolytic enzymes (e.g. calpains), presumably through the release of free Ca^{2+} ions from sarcoplasmic reticulum and other organelles and/or the release of cathepsins from disrupted lysosomes (Got *et al*. 1999).

Currently, few research workers consider the involvement of the caspase family in meat tenderisation and, in particular, with an additional stress such as ultrasound being applied.

The objectives of this study were to investigate the influence of ultrasound together with either a caspase-3 inhibitor, or a protease inhibitors cocktail, on myofibrillar protein degradation in chicken skeletal muscle. Such an approach with the use of inhibitors may allow the determination of the importance of specific endogenous proteases in combination with tissue disruption applied through sonication. To this end, we compared the protein degradation patterns among different treatments and control groups during post mortem aging, by measuring calpastatin activity, caspase-3/7 activity, shear force, and cooking loss.

MATERIAL AND METHODS

Chemicals. The following reagents were purchased from Amresco company (Amresco, Solon, USA): sodium dodecyl sulphate (SDS), Triton X-100, dl-dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris base), dimethyl sulfoxide (DMSO), dihydrate (EDTA Na_2), ethylenediaminetetra-acetic acid disodium salt, protease inhibitor cocktail tablet, Z-DEVD-fmk.

The following were purchased from other companies: BCA Protein Assay Kit (Bi Yuntian, Haimen, China), Polyclonal rabbit antibody (Bioss, Beijin, China), goat anti-rabbit IgG, HRP-linked antibody (Boster, Wuhan, China), Enhanced HRP-DAB kit (Tian gen, Beijing, China), and the end-point Apo-One Homogeneous Caspase-3/7 Assay (Promega, Madison, USA).

Samples and treatments. Six hens (culled, laying hens) of more than three years of age and weighing approximately 2.5 kg were selected for this work. Hens were handled and slaughtered according to the procedures approved by Anhui Academy of Agricultural Sciences. The breast meat was quickly removed and cut into small pieces (0.5 g/piece). The 0 day sample was obtained within 10 min and then stored at –80°C prior to subsequent analysis. Other muscles were divided into six groups and treated individually on the same day. The samples of Group A were soaked in a solution containing 2 mmol/l NaN_3 and 100 mmol/l NaCl using a meat: solution ratio of 1:1 (w/v) and were used as controls. The samples of Group B and Group C were soaked with the same solutions as the controls,

but these included 20 µmol/l caspase-3 selective inhibitor (Z-DEVD-fmk) protease inhibitor cocktail (Amresco M221), or protease inhibitor cocktail (Amresco M221) respectively. Group D samples were soaked in the same solutions as were those for C group (in order to eliminate most proteolytic enzymes, the role of ultrasound was uniquely determined), but were subjected to ultrasonics, using a 24-KHz ultrasonic probe placed at intervals on the meat surface for 15-s periods to expose the entire surface of the meat to 12 W/cm^2 of ultrasound, 4 minutes. Group E samples were soaked in the same solutions as were those for the controls, but these were subjected to ultrasonics using 24 KHz, 12 W/cm^2, 4 min (Jayasooriya *et al*. 2007). Group F samples were soaked in a mixture containing aprotinin (1 µg/ml), leupeptin (2 mmol/l), and 20% DMSO. All samples were then stored at 4°C for either 0, 1, 3 or 7 days. On each day, muscle samples were removed and stored at –80°C until required for analysis.

Sarcoplasmic protein preparation. Two grams of hen breast muscle was homogenised in 10 volumes (w/v) of 0.02 mol/l PBS (pH 6.5) at 6000 g for a total time of 3 minutes. A cooling period of 15 s was used between bursts. The homogenate was centrifuged at 6000× *g* for 20 min at 4°C, and the supernatant, containing sarcoplasmic proteins, was used for the determination of the relative calpastatin activity. Protein content was determined using the BCA Protein Assay Kit according to the instructions.

Myofibrillar protein extraction. The pellets obtained from the sarcoplasmic protein preparations given above were suspended in 10 volumes of 0.03 mol/l PBS (pH = 6.5) and centrifuged at 10 000 g and 4°C for 20 minutes. The pellets obtained were washed with 10 volumes of 0.03 mol/l PBS (pH 6.5), three times. The final pellet was suspended in 8 ml of 0.1 mol/l PBS (pH 6.5) containing 0.7 mol/l KI and 0.02% NaN_3 and homogenised at 7000 g for 3 min, the temperature being maintained at 4°C. After centrifugation at 10 000 g for 20 min at 4°C, the supernatant contained solubilised myofibrillar proteins. Protein content was determined using the BCA Protein Assay Kit, according to the instructions provided.

Gel system. Polyacrylamide separating gels of 12% and 10% (acrylamide: *N*,*N*'-bis-methylene acrylamide = 36.5:1 [w/w], 0.1% [w/v] SDS, 0.04% [v/v] TEMED, 0.1% [w/v] APS, and 0.375 mol/l Tris-HCl, pH 8.8) were used for the determination of the myofibril proteins changes in troponin-T and desmin, respectively. A 5% polyacrylamide gel (acrylamide:*N*,*N*'-bis-methelyene acrylamide = 36.5:1 [w/w], 0.1%[w/v] SDS, 0.1% [v/v] TEMED, 0.1% [w/v] APS, and 0.125 mol/l Tris-HCl, pH 6.8) was used as the stacking gel. A 5% polyacrylamide continuous gel (acrylamide: *N*,*N*'-bis-methylene acrylamide = 100:1 [w/w], 0.1% [w/v] SDS, 0.067% TEMED, 0.1% [w/v] APS, 2 mmol/l EDTA, and 200 mmol/l Tris-HCl, pH = 8.0) was used to determine the changes of nebulin.

SDS-PAGE. For the examination of desmin degradation, we used gels in a mini slab electrophoresis tank. The running buffer included 192 mmol/l glycine, 25 mmol/l tris, 0.1% (w/v) SDS, pH 8.3. Each lane was loaded with myofibrillar proteins (40 µg for desmin and 20 µg for troponin-T) and run at a constant voltage of 100 V for approximately 5 hours. With 5% gels, 80 µg of myofibrillar proteins were loaded. The gels were run on a Bio-Rad Mini-Protean II system at a constant current setting of 16 mA/gel for 5 hours. The gels were fixed for 4–12 h, stained overnight, fixed in 45% (v/v) methanol and 10% (v/v) glacial acetic acid, and stained for 1 h in an excess of 0.1% (w/v) Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) glacial acetic acid until the next day. The gels were then destained in an excess of the same solution not containing Coomassie brilliant blue R-250 and were kept in 10% (v/v) methanol and 10% (v/v) glacial acetic acid.

Western blotting. Gels for nebulin/desmin were rapidly transferred to PVDF membranes using a Mini-Protean II system at a constant current of 250 mA for 2.5 h at 4°C for nebulin and for 1.5 h at 4°C for desmin. The transfer buffer contained 192 mmol/l glycine, 25 mmol/l Tris, and 20% (v/v) methanol. The electro-blotted membrane was then blocked overnight in blocking buffer (TTBS: 10 mmol/l Tris, 150 mmol/l NaCl, 0.1% Tween 20, 5 mmol/l KCl, and 3% skim milk powder, pH 7.4) at 4°C. The PVDF membrane was incubated with polyclonal rabbit antibody to hen skeletal muscle protein desmin for 1 h at room temperature in the blocking buffer after blocking (a dilution of 1:500); and with polyclonal rabbit antibody to hen protein nebulin in the blocking buffer (dilution of 1:300, Bioss, Beijin, China). The PVDF membranes were incubated with goat anti rabbit IgG horseradish peroxidase (Boster, Wuhan) at a dilution of 1:10 000 in the blocking buffer after washing with TTBS 3–4 times, 5 min each. After the washing as above, the PVDF membrane was stained with a fast DAB (Enhanced HRP-DAB kit, Tiangen, China).

Semi-quantitative analysis of destained proteins. The gels or PVDF membranes were scanned (with a scanner, HP Scanjet G4010) at a resolution of 300 dpi, and the intensities of the ands were quantified using Scion Image software (Scan Band 5.0). Troponin-T degradation was identified through the increase in the intensity of the 30 kDa band and desmin degradation was shown through the reduction of intensity of the approximately 55 kDa band. The relative value of desmin degradation was calculated as the blot grey value of the desmin fragment in each gel over that of desmin at 0 day. The relative rates of troponin-T degradation product were calculated as the grey value of the 30 kDa fragment band in each gel over that of the actin in the same lane.

Heated (crude) calpastatin. Calpastatin activity of breast skeletal muscles was determined on days 0, 1, 3, and 7 after death according to Koohmaraie *et al*. (1995), with small modifications. In brief, 2 g of minced breast muscle was homogenised in 6 volumes of ice-cold extraction buffer (5 mmol/l EDTA, 50 mmol/l Tris-HCl, 1 mmol/l PMSF (phenylmethanesulfonylfl-uoride), 10 μmol/l of leupeptin, 50 mg/l of ovomucoid, pH 8.3) and centrifuged at 25 000 g for 1 h at 4°C. The supernatant was heated to inactivate calpain activity after dialysis, and then centrifuged at 25 000 g for 1 h at 4°C, and calpastatin was purified using a DEAE-Sephacel column (Koohmaraie *et al*. 1995). Calpastatin activity was determined from the inhibition of purified m-calpain activity by calpastatin extracted from the breast skeletal muscles. The results are reported as units of calpastatin activity per g of breast skeletal muscle; a unit is defined as the quantity of calpastatin required to inhibit 1 unit of m-calpain activity.

Shear force and cooking loss. The same meat samples were used for determining the cooking loss and shear force on days 0, 1, 3, and 7 *post mortem*. The stored samples were taken from the vacuum bags, blotted dry and weighed for the determination of drip loss:

$$\text{Drip loss} = \frac{\text{Initial weight} - \text{Drip weight}}{\text{Initial weight}} \times 100\%$$

followed by cooking in a water bath at 80°C until an internal temperature of 70°C was reached, then they were taken from the bags, dried with filter paper and then weighed. The cooking loss was then calculated as:

$$\text{Cooking loss} = \frac{\text{Drip weight} - \text{Cooking weight}}{\text{Drip weight}} \times 100\%$$

Followingly, the cooking samples were kept overnight at 4°C and Warner-Bratzler shear force (WBSF) was determined on each of them according to the method of Møller (1981); Shackelford *et al*. (2004), except that a square blade was used (12 mm width, 1.1 mm thickness).

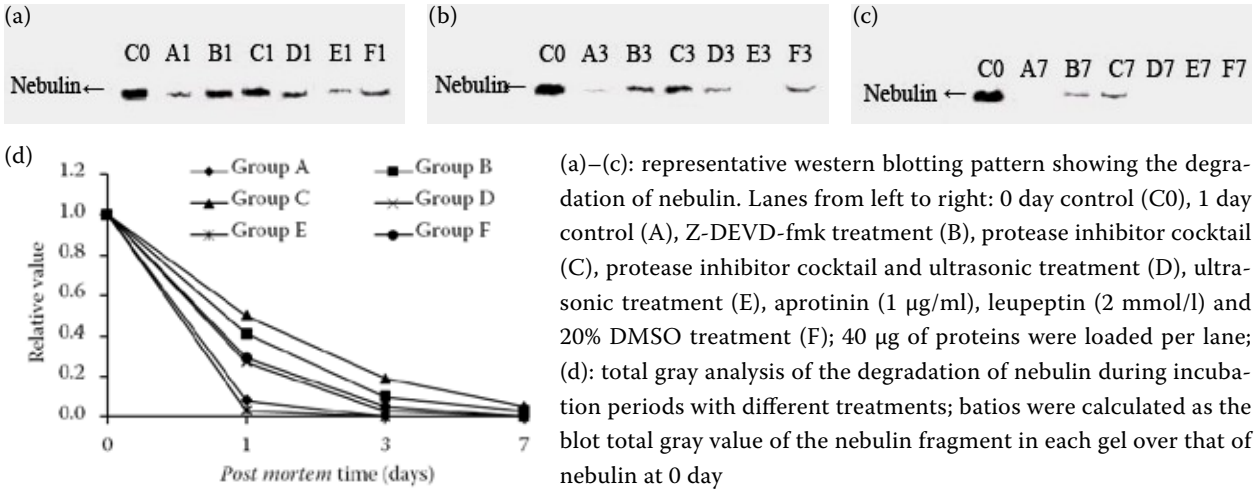
Determination of Caspase 3/7 activity. Caspase 3/7 activity was determined in breast muscle samples according to the method of Wagner *et al*. (2003) and Kemp *et al*. (2009). Frozen samples were crushed in liquid nitrogen, and 1 g was homogenised in 3 volumes of extraction buffer [25 mmol/l HEPES (pH = 7.5), 1 mmol/l EGTA, 1 mmol/l EDTA, 0.1% (v/v) Triton X-100, 5 mmol/l $MgCl_2$, 2 mmol/l DTT(1,4-dithiothreitol), (ethylene glycol tetra-acetic acid), 74 μmol/l antipain, 20 μmol/l leupeptin, 15 μmol/l pepstatin, 0.15 μmol/l aprotinin] for 2 × 20 seconds. The homogenate was centrifuged at 10 000× *g* and 4°C for 30 minutes. The supernatant was discarded and atoved and caspase-3/7 reagent was added to the supernatant at a 1:1 ratio. The reaction product was incubated at room temperature for about 7 hours. The intensity of fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with the use of a fluorospectro photometer (Turner Quantech Digital Filter Fluorometer, Iowa, USA).

Data analyses. The data obtained with the individual samples were analysed by single factor analysis of variance with the SPSS 17.0 statistical analysis software, and the differences between the individual means were compared with Duncan's multiple range test. The differences were considered significant at $P < 0.05$.

RESULTS

Protein degradation

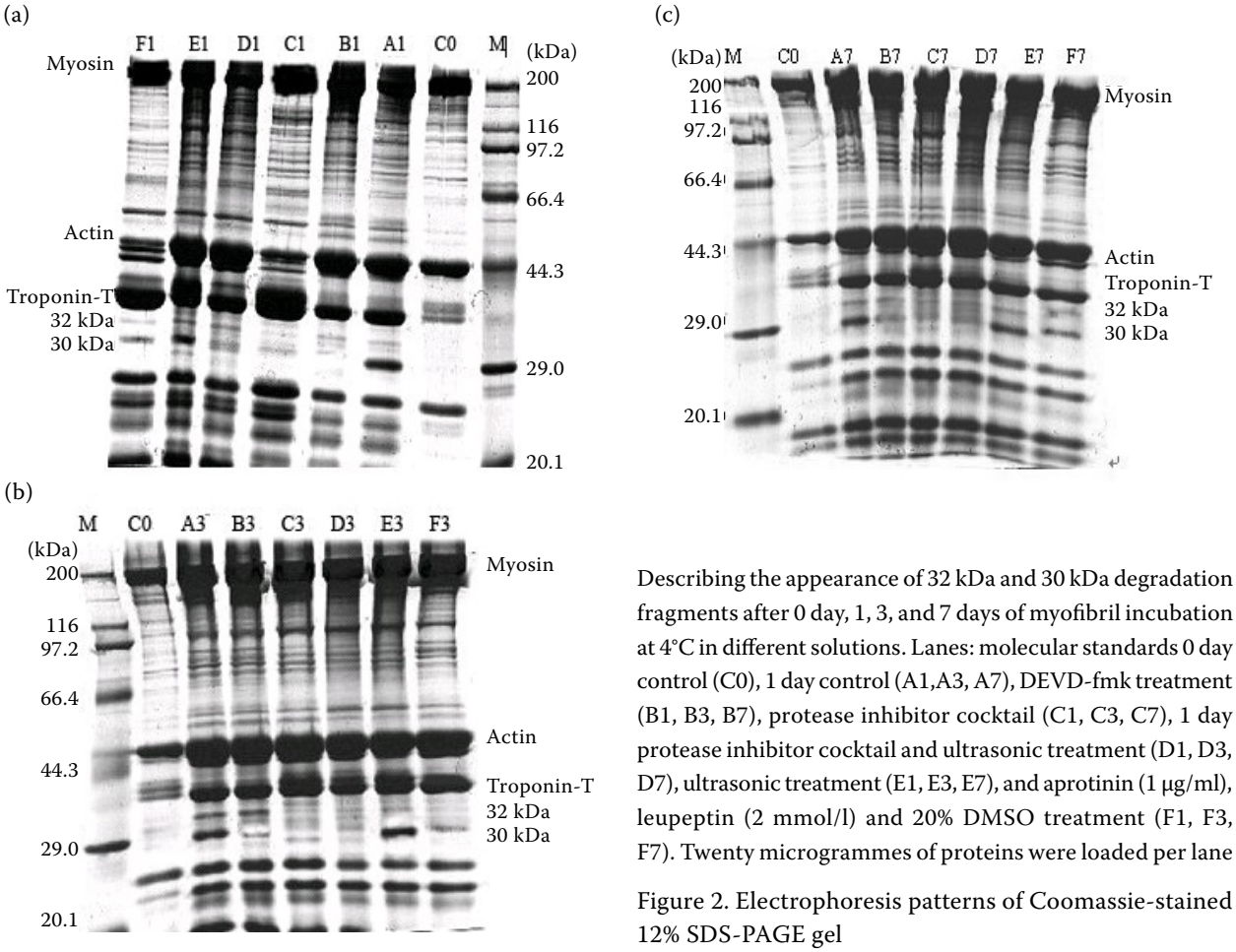
Nebulin. The *post mortem* degradation patterns of giant myofibrillar proteins such as titin and nebulin were examined after storage for 0, 1, 3, and 7 days at 4°C. The results, obtained on 5% continuous polyacrylamide separating gels demonstrated that the degradation of nebulin was effectively inhibited by the addition of exogenous enzyme inhibitors, caspase-3 inhibitor, and the protease inhibitor cocktail (Figure 1), with the intensity of the nebulin band decreasing gradually with time. About ninety-two percent of the nebulin in the



(a)–(c): representative western blotting pattern showing the degradation of nebulin. Lanes from left to right: 0 day control (C0), 1 day control (A), Z-DEVD-fmk treatment (B), protease inhibitor cocktail (C), protease inhibitor cocktail and ultrasonic treatment (D), ultrasonic treatment (E), aprotinin (1 μg/ml), leupeptin (2 mmol/l) and 20% DMSO treatment (F); 40 μg of proteins were loaded per lane; (d): total gray analysis of the degradation of nebulin during incubation periods with different treatments; batios were calculated as the blot total gray value of the nebulin fragment in each gel over that of nebulin at 0 day

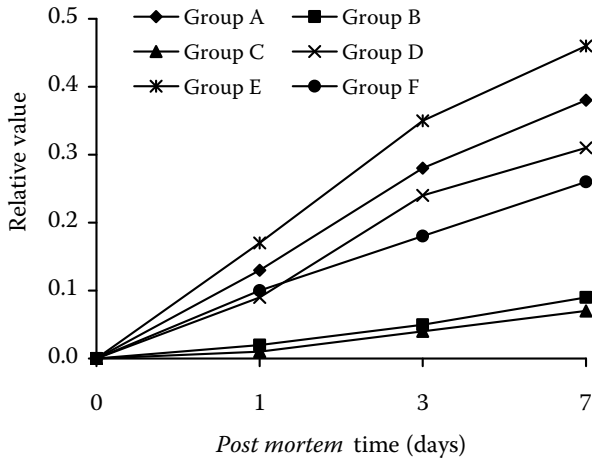
Figure 1. Changes of nebulin at 0, 1, 3, and 7 day *post mortem* of chicken breast muscle incubation at 4°C in different treatment

control sample (A) had been degraded by 1 day *post mortem*, and became difficult to see thereafter. In the ultrasound treated Group E, almost 97% of intact nebulin in the control samples were degraded. At the same time, 27% remained in Group D where ultrasound was used in the presence of caspase inhibitor; in Group B, treated with Protease Inhibitor cocktail, remained about 29%, whereas in Group C with the caspase selective inhibitor, DEVD-fmk-treated samples, approximately 41% of nebulin remained intact on day 1 *post mortem*. In the protease inhibitor cocktail-treated Group B, about 50% of nebulin remained intact on day 1 *post mortem*, and the initial band of nebulin could be



Describing the appearance of 32 kDa and 30 kDa degradation fragments after 0 day, 1, 3, and 7 days of myofibril incubation at 4°C in different solutions. Lanes: molecular standards 0 day control (C0), 1 day control (A1,A3, A7), DEVD-fmk treatment (B1, B3, B7), protease inhibitor cocktail (C1, C3, C7), 1 day protease inhibitor cocktail and ultrasonic treatment (D1, D3, D7), ultrasonic treatment (E1, E3, E7), and aprotinin (1 μg/ml), leupeptin (2 mmol/l) and 20% DMSO treatment (F1, F3, F7). Twenty microgrammes of proteins were loaded per lane

Figure 2. Electrophoresis patterns of Coomassie-stained 12% SDS-PAGE gel



Ratios were calculated as total gray value of the 30 kDa fragment in each gel over that of the actin within the same lane.

Figure 3. Total gray analysis of the appearance of the 30 kDa degradation fragment during different incubation periods with different solutions

seen even after 7 days *post mortem*. The extents of nebulin degradation between treatments with exogenous enzyme inhibitors and controls were significantly different at each time point (Figure 1).

Troponin-T. The *post mortem* degradation of troponin-T and the existence of 30 kDa fragment have been reported frequently (Ho *et al*. 1996; Negishi *et al*. 1996;). In the present research, 12% polyacrylamide separating gels revealed a difference between the different treatment groups in the existence of 30 kDa and 32 kDa degraded fragments (Figure 2). On the first day *post mortem*, the 30 kDa and the 32 kDa bands could be also readily observed in the control samples (Figure 2). The temporal change of 30 kDa degradation products was obvious. Total grey analysis indicated that the grey value of the 30 kDa band over that of actin in the control sample in the same lane on days 1, 3, and 7 *post mortem* was 13, 28 and 38%, respectively (Figure 3). However, compared to the control, there was a considerable decrease in the ratio of troponin-T degradation in the treated samples. With the addition of protease inhibitor cocktail-treated (Group B) and the DEVD-fmk treated samples (Group C), the 30 kDa band could not be readily detected until 7 days *post mortem*, and only then, it accounted for 9% and 7% of that of actin. This is in accordance with the reports by others (Huang *et al*. 2009). In the ultrasound treated Group D, the rate of troponin-T degradation was increased, and on day 7 *post mortem*, it amounted to 46%, in Group D while Group F also came up to 31% and 26%, respectively. At each of the experimental time points, the differences in the 30 kDa degraded fragments between protease inhibitor cocktail-treated and control samples, the DEVD-fmk treated, and control samples were significant ($P < 0.01$). The differences between the ultrasound-treated and control samples were also significant ($P < 0.05$).

Desmin. The degradation of desmin during *post mortem* aging was examined using a polyclonal antibody. Western blot analysis showed that the treatment with specific inhibitors of endogenous enzymes had significant effects on the degradation of desmin (Figures 4 and 5). In summary, it was obvious that the protease inhibitor cocktail-treated Group B and DEVD-fmk treated samples (C) showed slower degradation of intact desmin than did the control. On days 1, 3, and 7 *post mortem*, in the control samples nearly 61, 85, and almost 100% of intact desmin disappeared while in the cocktail-treated samples, it was about 82, 56, and 51%, respectively. In the DEVD–fmk-treated samples, approximately 75, 49, and 45% of desmin remained intact at each of the three time points, respectively (Figures 4 and 5). In Group D and Group F, the degradation also was a little slower than in the control samples. Only the ultrasonic-treated samples showed faster

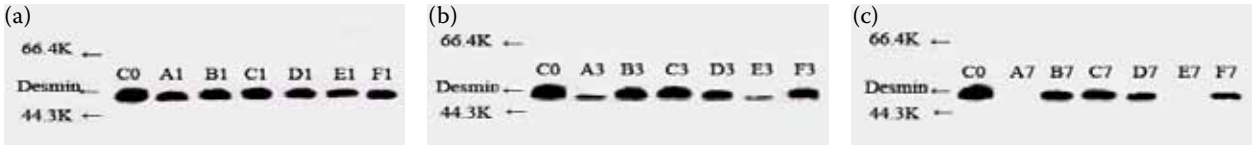
Table 1. Mean ± SEM for calpastatin activity of different treated chicken meat when stored at 4°C for 0, 1, 3, and 7 days during *post mortem* storage[1]

Post mortem time (day)	Group A	Group B	Group C	Group D	Group E	Group F
0	1.99 ± 0.15^{a}	2.05 ± 0.27^{a}	2.12 ± 0.45^{a}	1.68 ± 0.31^{a}	2.04 ± 0.22^{a}	1.71 ± 0.08^{a}
1	0.85 ± 0.09^{a}	1.47 ± 0.10^{bc}	1.71 ± 0.16^{c**}	1.29 ± 0.11^{c*}	0.79 ± 0.10^{a}	1.21 ± 0.05^{c*}
3	0.45 ± 0.05^{a}	0.81 ± 0.05^{bc}	0.92 ± 0.14^{c**}	0.67 ± 0.04^{c*}	0.39 ± 0.15^{a}	0.71 ± 0.05^{c*}
7	0.27 ± 0.02^{a}	0.79 ± 0.03^{b}	0.68 ± 0.05b^{c**}	0.47 ± 0.01^{d*}	0.20 ± 0.03^{a}	0.45 ± 0.04^{d*}

[1]units are total calpastatin activity per gram of muscle

$^{a–c}$means within the same row with different superscripts are different ($P < 0.05$)

*$P < 0.05$ and **$P < 0.01$ means between different treatment groups are significant

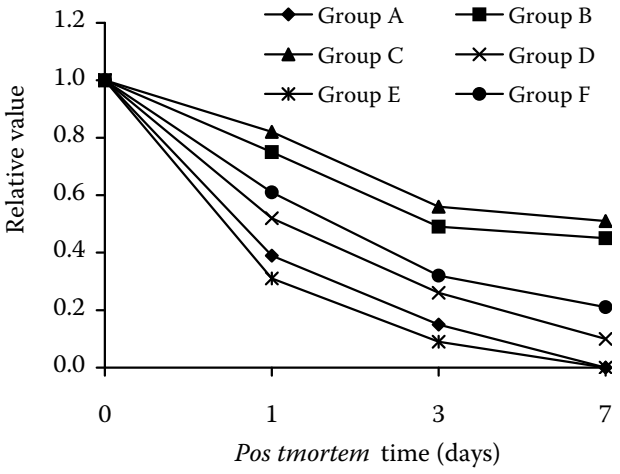


(a)–(c): representative western blotting pattern showing the degradation of desmin. Lanes from left to right: molecular standards, 0 day control (C0), 1 day control (A1, A3, A7), DEVD-fmk treatment (B1, B3, B7), protease inhibitor cocktail treatment (C1, C3, C7), protease inhibitor cocktail and ultrasonic treatment (D1, D3, D7), ultrasonic treatment (E1, E3, E7), and aprotinin (1 μg/ml), leupeptin (2 mmol/l) and 20% DMSO treatment (F1, F3, F7)

Figure 4. Changes of desmin after 0, 1, 3, and 7 days of chicken breast muscle incubation at 4°C in different solutions

degradation. During 7 days of aging, the difference in desmin degradation between protease inhibitor cocktail-treated and control samples was statistically significant ($P < 0.01$). This was also the case for the differences between DEVD-fmk treated and control samples ($P < 0.01$), and between Group F samples and control samples ($P < 0.05$).

Heated (crude) calpastatin. On days 0, 1, 3, and 7 *post mortem*, calpastatin activity was determined in hen breast muscles (Table 1), showing that the residual activity with ageing was higher in the protease inhibitor cocktail-treated samples compared with the control samples on each day of measurement ($P < 0.01$). The rate of decrease in the calpastatin activity during *post mortem* storage was faster in the control samples compared with protease inhibitor cocktail-treated samples.



Forty microgrammes of proteins were loaded per lane. Intensity analysis of the degradation of desmin during incubation periods with different treatments. Ratios were calculated as the blot total gray value of the desmin fragment in each gel over that of desmin at 0 day

Figure 5. Desmin changes of after 0, 1, 3, and 7 days of chicken breast muscle incubation at 4°C in different solutions

In Group F, calpastatin activity was higher than that measured in the control samples. There was little difference between the DEVD-fmk-treated and the control samples. In Group D, calpastatin activity was higher than in the control samples, and somewhat lower than that in protease inhibitor cocktail-treated samples (Group B). This may be attributed to ultrasonic treatment which may not only promote the release of cathepsins, but also allow the access of protease inhibitor to the endogenous enzymes. It can be expected that, when the concentration of protease inhibitor is higher than that of protease, the action of cathepsins may be inhibited and protein degradation reduced.

Shear force and cooking loss. Overall, the shear force values in cooked hen breast meat were the highest on the 1st day and lowest after 7 days, falling by about 1.5 kg during this period. The shear force values in the ultrasonic treatment group (Group E) were lower ($P < 0.01$) compared with the control samples (Table 2). The shear force values in the samples of Group D were also lower ($P < 0.05$) compared with the control samples, while there were no significant differences between the cooking loss in ultrasonic treatment group and that for the incorporating treatment group. The shear forces and cooking losses were both lower in Group D compared with the control (Group A) ($P < 0.05$). A negative correlation between the shear force and cooking loss over the *post mortem* storage period was detected ($P < 0.05$, $R^2 = 0.91$, $R = 0.95$), together with a positive correlation between the shear force and calpastatin activity ($P < 0.01$, $R^2 = 0.65$, $R = 0.81$).

Caspase-3/7 activity. Caspase-3 activity was measured by a standard fluorescence-based assay which cannot distinguish between caspases-3 and caspases-7 activities (Chen *et al*. 2011). The caspase-3/7 activity increased within 24 h, thus the caspase-3/7 activity on day 1 was the highest in all treatments except for the DEVD-fmk-treated group. However, no further significant variation

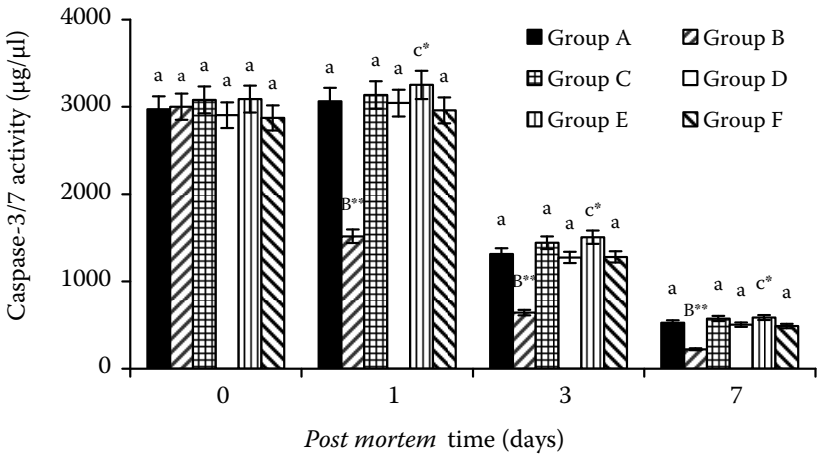


Figure 6. Caspase-3/7 activities of different treated chicken meat when stored at 4°C for 0, 1, 2, 3 and 7 days

Data are representatives of four independent experiments and are presented as means ± SD; different small and capital superscipts in the same row mean different significantly at $P < 0.05$ and $P < 0.01$ levels, respectively

*$P < 0.05$ and **$P < 0.01$ means between different treatment groups are significant

was observed after 3 days or 7 days in any of the six groups, and the rate of decline remained more or less constant during 7 days of ageing, except for the DEVD-fmk-treated group (Table 3). Compared to the control, the caspase-3/7 activities on ultrasonic treatment were significantly higher on the 1st day ($P < 0.05$), their activity having been increased directly through the physical weakening of the muscle structure, or indirectly through the activation of proteolysis, either by the release of cathepsins from lysosomes and/or of Ca^{2+} ions from intracellular stores. During the 7 days *post mortem*, a decreasing trend was observed for caspase-3 activity of the control, but differences were not significant except for the DEVD-fmk-treated samples. This may have resulted from the influence of protease inhibitor on caspase-3/7 activity. In addition, in the combined treatment samples (Group D), the caspase-3/7 activity was higher than in the control samples, which may be attributed to ultrasonic treatment, where ultrasound may have accelerated the release of caspase-3/7, thus increasing its activity.

DISCUSSION

Many reports have indicated that proteolytic degradation of the skeletal muscle proteins nebulin, troponin-T, desmin, vinculin, and filamin, plays a key role in the tenderness of aged meat.

The rate of muscle degradation and *post mortem* tenderisation are highly dependent on the endog-

Table 2. Kramer shear force value (kg) of different treated chicken breast meat when stored at 4°C for 0, 1, 3, and 7 days

Post mortem time (day)	Group A	Group B	Group C	Group D	Group E	Group F
0	4.97 ± 0.62^{a}	4.85 ± 0.44^{a}	5.11 ± 0.37^{a}	5.04 ± 0.58^{a}	4.92 ± 0.74^{a}	4.84 ± 0.91^{a}
1	4.51 ± 0.29^{a}	5.07 ± 0.52^{b}	4.65 ± 0.15^{ab}	$3.97 \pm 0.30^{cd*}$	$3.59 \pm 0.09^{d**}$	4.73 ± 0.47^{a}
3	3.82 ± 0.11^{a}	$4.26 \pm 0.69^{b*}$	$4.39 \pm 0.36^{b*}$	$3.31 \pm 0.19^{c*}$	$2.84 \pm 0.45^{d**}$	$4.36 \pm 0.66^{b*}$
7	3.48 ± 0.34^{a}	$4.14 \pm 0.22^{b*}$	$4.25 \pm 0.16^{b*}$	$2.79 \pm 0.41^{c*}$	$2.29 \pm 0.39^{d**}$	$4.28 \pm 0.26^{b*}$

Different small and capital superscipts in the same row mean different significantly at $P < 0.05$ and $P < 0.01$ levels, respectively

Table 3. Caspase-3/7 activities of different treated chicken meat when stored at 4°C for 0, 1, 2, 3, and 7 days

Post mortem time (day)	Group A	Group B	Group C	Group D	Group E	Group F
0	2971 ± 212^{a}	3002 ± 169^{a}	3079 ± 338^{a}	2904 ± 156^{a}	2988 ± 319^{a}	2873 ± 152^{a}
1	3064 ± 305^{a}	$1517 \pm 176^{b**}$	3135 ± 402^{a}	3194 ± 236^{a}	$3352 \pm 298^{c*}$	2958 ± 77^{a}
3	1315 ± 126^{a}	$643 \pm 97^{b**}$	1444 ± 179^{a}	1274 ± 76^{a}	1607 ± 124^{a}	1281 ± 132^{a}
7	527 ± 82^{a}	$221 \pm 43^{b*}$	575 ± 72^{a}	504 ± 64^{a}	586 ± 113^{a}	488 ± 74^{a}

Values are expressed as absorbance (arbitrary units) per µg/ul protein concentration; each value represents the mean ± SD

$^{a-d}$means within a row with different superscripts are significantly different($P < 0.05$)

*$P < 0.05$ and **$P < 0.01$ means between different treatment groups are significant

Table 4. The cook loss of different treated chicken breast meat when stored at 4°C for 0, 1, 3, and 7 days

Postmortem time (day)	Group A	Group B	Group C	Group D	Group E	Group F
0	27.25 ± 0.98	28.07 ± 0.46	27.69 ± 0.42	27.01 ± 0.56	26.84 ± 0.77	27.83 ± 0.42
1	29.67 ± 1.24^{a}	29.24 ± 0.33^{a}	28.71 ± 0.64^{a}	29.98 ± 0.66^{a}	32.39 ± 0.29^{b}	28.94 ± 0.99^{a}
3	32.41 ± 0.37^{a}	29.97 ± 0.75^{b}	29.31 ± 0.69^{c}	30.84 ± 0.97^{abd}	33.73 ± 1.04^{a}	29.21 ± 0.34^{bcde}
7	35.98 ± 0.65^{a}	29.68 ± 0.51^{B}	29.46 ± 0.57^{BC}	31.71 ± 0.19^{BCd}	34.52 ± 0.48^{a}	30.06 ± 0.79^{bcdE}

Different small and capital superscipts in the same row mean different significantly at $P < 0.05$ and $P < 0.01$ levels, respectively

enous enzymes. A decrease in muscle protein degradation by protease inhibitors has been shown to promote muscle growth. This strongly demonstrates that endogenous enzyme inhibitors have a key role; for example calpastatin, a calpain inhibitor, cystatins, a family of cysteine and serine protease inhibitors. However, apart from calpastatin (Sentandreu *et al*. 2002), a specific inhibitor, little is known about the potential endogenous inhibitors of the other proteolytic enzymes in animal skeletal muscle.

The application of Protease Inhibitor Cocktails, AEBS, Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A, and Calpain inhibitor 1 on buffalo meat has suggested that higher calpain activities are responsible for the increased tenderness of water buffalo meat compared to beef soon after slaughter (Neath *et al*. 2007).

However, at this time, there is no absolute proof that apoptosis and caspases play a role in skeletal muscle *post mortem* proteolysis. In the current study, when the inhibitor of caspase-3 DEVD-CHO was added into the soaking solution, the degradations of nebulin, titin, troponin-T, and desmin appeared to be significantly inhibited (Huang *et al*. 2009). Troponin-T is not thought to be an important protein in the maintenance of structure, but the 30 kDa fragment of troponin-T is believed to be an indicator of aging and tenderisation of skeletal muscle (Huff-Lonergan 1996a; Huang *et al*. 2009). Chen *et al*. (2011) reported that the three apoptosis inducers used in their study all increased myofibrillar protein degradation and proteolysis during the first 3 days of chicken meat ageing as a result of high activity of caspase-3/7, and they argued that it is therefore possible that caspase-3 contributes to the conversion of muscle into meat. In addition, other studies have demonstrated that effector caspases can decompose skeletal muscle proteins, such has actin, desmin, and spectrin (Du *et al*. 2004; Chen *et al*. 2011). To evaluate further whether different exogenous proteolytic enzymes inhibitors influence the activity of calpastatins, we analysed the activity of calpastatin. The results showed that there was no statistical difference between DEVD-fmk-treated and control samples during the whole incubation period, but statistical difference existed between protease inhibitor cocktail-treated and the control samples ($P < 0.01$).

Therefore, the retarded degradation of skeletal proteins was attributed to the calpastatin activity in the protease inhibitor cocktail-treated samples (Group B), while that of skeletal proteins cannot be attributed to the calpastatin activity in the caspase-3 inhibitor-treated samples (Group C). This is in accordance with the findings of Huang *et al*. (2009) where the activity of calpain was little affected with the use of caspase-3 inhibitors.

Caspases have been shown to be active during the early ageing period, that their activities correlating with shear force (Kemp *et al*. 2006). Caspases may play a role in tenderisation through modulating the calpain system, most likely by degrading the calpain specific inhibitor calpastatin. A number of studies have demonstrated that caspases are capable of cleaving calpastatin resulting in an increase in calpain activity.

Kemp *et al*. (2006) reported that there was a negative relationship between shear force and the 0 h to 32 h ratio of caspase-3/7 ($r = -0.62$, $P = 0.053$). In our studies, we found that as shear force increased, caspase-3/7 activities decreased from 0 day to 1 day, which is in accordance with the results of Kemp *et al.* (2006). A negative relationship was also detected between the peak caspase-3/7 activity at 8 h in longissimus muscles from normal lambs and calpastatin activity at 0 day and 2 days ($r = -0.65$, $r = -0.68$, respectively, $P < 0.05$). Again, in our experiment, calpastatin activity decreased with increased time, as found by Kemp *et al*. (2009). This is therefore evidence for the indirect involvement of caspase in *post mortem* proteolysis and meat tenderisation, although other studies suggest a direct role for caspases and apoptosis in *post mortem* proteolysis.

Others researchers have suggested ultrasonic tenderisation mechanisms which include lysosomal rupture and disruption of the myofibrillar proteins or connective tissues (Lyng & Allen 1997). The applications of ultrasound in meat tenderisation are well established. The intrinsic properties of the meat can be altered in a sufficiently high power range (20–100 kHz, 100 W–10 kW). Jayasooriya *et al*. (2007) have reported that raw beef samples can be tenderised when treated with high power ultrasound (24 kHz, 12 W/cm^2) for 240 seconds.

Jayasooriya *et al*. (2007) found that ultrasound treatment significantly reduced WBS force and hardness, but the drip, cooking and total losses were not affected by ultrasound treatment in their experiment. This suggests that the damaged muscle structure caused by ultrasound did not affect the mechanism of moisture migration in meat, and our results are consistent with this finding. In contrast, Pohlman *et al*. (1997) concluded that ultrasound treatment of meat could reduce drip loss. However, in their study the samples were vacuum packaged and exposed to high-intensity ultrasound, whereas in our work meat was stored at 4°C *post mortem*, which may account for the differences.

Consequently, the use of ultrasound combined with enzymes inhibitors should be further investigated.

CONCLUSIONS

The current results have showed that the protease inhibitor cocktail, the selective inhibitor of caspase-3 DEVD-fmk and aprotinin, leupeptin, markedly inhibited the degradation of skeletal muscle proteins, titin, nebulin, desmin, and troponin-T, which are degraded naturally in *post mortem* skeletal muscles. Moreover, the activity of calpain was not affected by the addition of DEVD-fmk. Therefore, the degradation of muscle proteins should not be exclusively attributed to the calpain system, and the effector caspase-3 may be involved in *post mortem* muscle protein proteolysis.

Exogenous enzyme inhibitors were found to inhibit the degradation of muscle proteins and decrease the cooking loss of hen meat, and further, ultrasound decreased the shear force of meat (increased tenderness), thus improving meat quality of culled, laying hens. The use of ultrasound did not significantly result in any decrease in the cooking loss and the combined treatments of ultrasonics and exogenous proteolytic enzyme inhibitors also could improve chicken meat tenderness and decrease the cooking loss.

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