# Postmortem role of calpain in Chinese and Wuzong goose muscles

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**ABSTRACT** The purpose of this study was to compare postmortem proteolysis and tenderization between Chinese and Wuzong goose breast muscles. Four months old Chinese (CG, n = 15) and Wuzong (WZ, n = 15) goose carcasses were vacuum-packaged 10 to 15 min postmortem and stored at 5°C. Breast (*Pectoralis major*) samples from each carcass were sampled at 0 (~10 min postmortem), 1, 3, and 7 D of storage. Our results showed that the decrease in pH and calpain-1 activity was not different in CG and WG samples. However, the decrease in calpain-11 activity, desmin content, and shear force were more rapid (P < 0.05) in WZ than in CG samples. Our results indicate that postmortem proteolysis and tenderization of goose breast muscle were more extensive in WZ than in CG goose muscle.

Key words: goose muscle, postmortem proteolysis, tenderization, calpain

2019 Poultry Science 98:7151–7157 http://dx.doi.org/10.3382/ps/pez515

#### INTRODUCTION

Previous studies have shown that the calciumdependent proteases (calpains) play a key role in proteolysis and tenderization of muscle during the first 7 to 14 D of postmortem storage at refrigerated temperature (Hopkins and Thompson, 2002; Koohmaraie and Geesink, 2006; Bhat et al., 2018). The 2 most studied ubiquitous calpain isoforms, calpain-1 (u-calpain), and calpain-2 (m-calpain), are present in mammalian muscle cells (Goll et al., 2003). However, Sorimachi et al. (1995) showed that there was little calpain-2 (m-calpain) mRNA expression, and no apparent calpain-2 activity in chicken skeletal muscle. Sorimachi et al. (1995) also reported that, rather than calpain-1 or calpain-2, calpain-11 (µ/m-calpain), whose sequence homology and Ca<sup>2+</sup>-sensitivity is between mammalian calpain-1 and calpain-2, was the predominant isoform in chicken skeletal muscle. Previous studies also showed that calpain-1 activity decreased very rapidly in postmortem chicken (Chang and Chou, 2010) and duck (Chang and Chou, 2012) muscles and was highly correlated with desmin and troponin-T degradation in postmortem duck muscle. In the absence of calpain-1 activity, very minor postmortem proteolysis and tenderization occurred in ostrich muscle stored at refrigerated temperature although autolyzed calpain-11 activity could be observed. In the presence of sufficient  $Ca^{2+}$ , however, calpain-11 did play a role in postmortem proteolysis and tenderization of ostrich muscle without the presence of calpain-1 (Chang et al., 2018).

Animal breed is an important factor impacting postmortem calpain-mediated proteolysis and tenderization in bovine (Pringle et al., 1997; Kuber et al., 2004; Monsón et al., 2005; Marino et al., 2013) and in duck (Liao and Chou, 2014) muscles. Chinese (CG) and Wuzong (WZ) geese are two popular meat sources in Asia. Previous studies have shown that postmortem tenderness of goose muscle is the lowest when compared with chicken, duck, and guinea fowl muscles (Geldenhuys et al., 2014), and meat gualities could be largely affected by goose breed (Okruszek et al., 2008, 2012). However, the contribution of calpain during postmortem aging of goose muscles in both CG and WZ, which are genetically different (Tu et al., 2006; Ren et al., 2016), has not been studied. It may be required to understand whether this breed difference has an impact on calpain changes in postmortem goose muscle. Therefore, the purpose of this study was to compare postmortem calpain-mediated proteolysis and tenderization between CG and WZ goose breast muscles.

# MATERIALS AND METHODS

## Sample Preparation

This study was approved by the Institutional Animal Care and Use Committee of National Chiayi University. Chinese geese and Wuzong geese were raised in a commercial poultry farm. All birds were fed ad libitum on standard commercial goose diets. After resting overnight with free access to drinking water,  $\sim$ 4-months-old female Chinese geese (n = 15, average

<sup>@</sup> 2019 Poultry Science Association Inc.

Received March 12, 2019.

Accepted August 30, 2019.

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live weight = 3.15 kg) and Wuzong geese (n = 15, average live weight = 2.95 kg) were harvest in a government-regulated abattoir via standard commercial practice, which met the requirement of hazard analysis and critical control points protocols. The samples size used in this study (n = 15) was comparable to that used in other similar calpain studies including n = 10to 12 in cattle studies by Pringle et al. (1997), n = 10in goat studies by Gadiyaram et al. (2008), n = 6 in swine studies by Rosell and Toldrá (1998), and n = 6 in duck studies by Liao and Chou (2014). The geese were electrically stunned, bled, and defeathered. After eviscerating and washing with  $\sim 15^{\circ}$ C water for 2 min to decrease the carcass temperature, the carcasses ( $\sim 10 \text{ min}$ postmortem) were then individually vacuum-packaged and stored at 5°C in a walk-in cooler. Breast muscle (*Pectoralis major*) from the left side of each carcass was randomly divided into 4 parts and sampled (50 to 60 g per each time point) at 0 ( $\sim$ 10 min postmortem), 1, 3, and 7 D of postmortem storage for the analysis of pH, casein zymography, and Western blot. The samples were finely minced, quickly snap-frozen in liquid nitrogen and stored at  $-80^{\circ}$ C until needed for subsequent analysis. The right side of the breast muscle was cut perpendicularly to the muscle fibers into 2 equal parts and randomly taken for 1- and 7-day samples ( $\sim 6 \times 4 \times$ 3 cm) of Warner-Bratzler shear force measurement.

## Measurement of pH

The pH measurement was done by the method of Yu and Lee (1986). All steps were conducted at 0 to 2°C to minimize the metabolic reactions for early postmortem samples as described by Li et al. (2012). After a 1-g sample was homogenized in 10 mL of pre-cold distilled water (0 to 2°C), the pH of the homogenate was determined with a pH meter (Model 470, Suntex Instruments Co., Taiwan) equipped with a glass electrode. Before measuring, the electrode was calibrated with pH 7.00 and with pH 4.01 buffer solutions at 25°C.

## Casein Zymography

Calpain extraction was done by the method of Veiseth et al. (2001) and carried out at 2°C in a cold room. Briefly, a 5-g sample was homogenized in 15 mL of extraction buffer [10 mM EDTA, 0.05% 2mercaptoethanol (MCE) (v/v), and 100 mM Tris base, pH 8.3]. After centrifuging at  $22,000 \times q$  for 25 min, protein concentration of the supernatant was measured by the method of Robson et al. (1968). A sample buffer that contained 0.05% MCE (v/v), 0.02% bromophenol blue (w/v), 20% glycerol (w/v), and 150 mM Tris-HCl, pH 6.8, was added to the protein extract at a ratio of 2 parts of the buffer to 3 parts of protein extract (v/v). Casein gels were routinely run in 10% gels (acrylamide: methylenebisacrylamide = 37.5:1, w/w) that contained 0.21% case in (w/v) by the method of Raser et al. (1995). Casein minigels (0.75 mm, Bio-Rad

Laboratories, Herculues, CA) were pre-run with a running buffer [0.05% MCE (v/v), 1 mM EDTA, 192 mM glycine, and 25 mM Tris-HCl, pH 8.3] at 100 V for 15 min. An aliquot of 250 µg of protein from each goose sample and a pooled 0-day CG sample, which was the standard reference, were loaded onto the casein gels. After running at 100 V for 2 h at 5°C, the casein gels were incubated at 25°C in 3 changes of a 50 mM Tris-HCl (pH 7.5) buffer that contained 0.05% MCE (v/v) and CaCl<sub>2</sub> (0.01, 0.03, 0.1, or 4 mM) with slow shaking for 1 h and then followed by a 16-h incubation in the same buffer at 37°C. The gels were stained with Coomassie blue and destained with 20% methanol (v/v) and 7% acetic acid (v/v) solutions.

## Western Blot Analysis

Breast myofibrils were prepared by the method of Huff-Lonergan et al. (1995). The myofibril protein concentration was determined by the method of Robson et al. (1968). Myofibril samples for SDS-PAGE were prepared by the method of Fritz and Greaser (1991). The SDS-PAGE procedures for myofibrils on 12%slab gels (acrylamide: methylenebisacrylamide = 37.5: 1, w/w) was done via the method of Laemmli (1970). The same amount of protein (250  $\mu$ g) from each sample including a pooled 0-day CG sample, which was used as the standard reference, was loaded onto each gel. A pre-stained marker that contained 9 polypeptides in the range of 4 to 250 kDa (SeeBlue Prestained Standard, LC5625, Invitrogen Co., Carlsbad, CA) was used as a protein standard. SE 400 slab gel electrophoresis units (Hoefer Scientific Instruments, San Francisco, CA) were used to run all gels with 15 mA at 25°C. After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane. Western blot analysis was done by the method of Liao and Chou (2014). A monoclonal primary antibody to desmin (Clone DE-U-10, 1:500 dilution, Sigma-Aldrich Company, St. Louis, MO) and a secondary antibody (goat anti-mouse-HRP, 1:1,000 dilution, Sigma-Aldrich Company) was used to label the blots. Color was developed by using SIGMAFAST 3,3'-diaminobenzidine tablets (Sigma-Aldrich Company).

## Image Analysis

Image analysis was performed by the method of Chang et al. (2013). Briefly, the bands in each blot or casein gel that had been incubated in 4 mM Ca<sup>2+</sup> from each goose sample were digitized with a scanner (Epson Perfection 4990 scanner, Model J131B, Epson America, Inc., Long Beach, CA). The resulting signals were quantified by Image J (version 1.44i, made by Wayne Rasband, National Institutes of Health, MD). Each blot or casein gel included a pooled 0-day CG sample as a reference standard to normalize the band intensities. The total calpain activity (summation of calpain-1, calpain-11 and putative calpain-2) and desmin content

6.50

in 0-, 1-, 3-, and 7-day of CG or WZ breast muscle samples were expressed as a percent of the 0-day WZ breast muscle samples, which was taken as 100%.

#### Warner–Bratzler Shear Force Measurement

Shear force measurement was done by the method of Vieira and Fernández (2014) and Chang et al. (2016). The 1- and 7-day samples were placed in a polypropylene bag and heated in an 85°C water bath until an internal temperature of 71°C was reached as measured with a temperature probe (Hanna Instruments, Woonsocket, RI) in the thickest part of the sample. After cooling in running tap water for 30 min, the samples were refrigerated at 4°C overnight. Eight subsamples (1 cm<sup>3</sup> cubes) were cut for Warner-Bratzler shear force determination. The cubes were sheared perpendicular to the fiber at a crosshead speed of 5 mm/s by a Texture Analyzer (Model TA-XT-plus, Stable Micro Systems Ltd., Godalming, UK) equipped with a Warner-Bratzler blade (code HDP/BS). The value reported for each sample was an average of the 8 cubes evaluated.

### Statistical Analysis

A split-plot design was used in this study. Whole units were the goose breed (CG and WZ), and subunits were the muscle samples taken at each sampling time. Data were analyzed by using the mixed model procedure (PROC Mixed) of SAS software. The fixed effects included goose breed, postmortem time and their interaction (breed  $\times$  time), and the random effects were geese. Tukey's test was used to separate multiple means at a 5% significance level.

## **RESULTS AND DISCUSSION**

### Postmortem pH Changes

Figure 1 shows that the postmortem mean pH was not different at each sampling time for CG and WZ goose breast samples. However, the pH in 0-day CG  $(6.11 \pm 0.06)$  and WZ  $(6.12 \pm 0.02)$  samples decreased significantly (P < 0.05) in 1-day CG ( $5.89 \pm 0.01$ ) and WZ ( $5.88 \pm 0.03$ ) samples. However, the pH remained nearly constant in 3-day ( $5.87 \pm 0.03$  CG vs.  $5.89 \pm$ 0.01 WZ) and in 7-day ( $5.89 \pm 0.02$  CG vs.  $5.89 \pm$ 0.01 WZ) samples. These results showed that the rate of postmortem pH decline was not affected by goose breed, confirming the previous studies of Okruszek et al. (2008) and Uhlířová et al. (2018), who observed no influence of goose breed on postmortem pH in breast muscle stored at 5°C.

## Postmortem Calpain Changes

Previous studies demonstrated that case zymography can be used to separate and identify calpain iso-



forms in avian muscle (Lee et al., 2007; Chang and Chou, 2012; Chang et al., 2013). In good agreement with previous studies (Chang et al., 2013), Figure 2 shows 3 calpain isoforms present in CG (Figure 2A) and WZ (Figure 2B) samples, namely calpain-1 (upper row), putative calpain-2 (middle row), and calpain-11 (bottom row). Moreover, a faint band just below the calpain-11 band in 1-day CG and WZ samples began to appear and was clearly seen in 3- and 7-day samples on the casein gels (Figure 2), indicating that an autolyzed calpain-11 band (the lower band) was generated after calpain-11 activation, consistent with previous studies in postmortem duck (Chang and Chou, 2012), ostrich (Chang et al., 2016), and chicken (Soglia et al., 2018) muscles.

When total calpain activity (summation of calpain-1, calpain-11 and putative calpain-2 activities) in 0day WZ samples was taken as 100%, the total activity in the 0-day CG (103%) and WZ (100%) samples was not different (P > 0.05) (Figure 3). Because the putative calpain-2 activity (no more than 3% of total calpain activity) in both CG and WZ samples (Figure 3) was very minor, it was reasonable to assume that the activity would not cause a notable effect on proteolysis and tenderization in postmortem CG and WZ muscle. On the other hand, the calpain-1 activity (Figure 3A) in 0-day CG samples (22% of total calpain activity) decreased rapidly to 7%, to 3%, and to 3% of the total activity in 1-day, 3-day, and 7-day samples, respectively. Similarly, the 0-day WZ calpain-1 activity (20% of total calpain activity) decreased to 4%, to 3%, and to 2% of the total activity in 1-day, 3day, and 7-day samples, respectively (Figure 3B). These results showed that calpain-1 activity was not different (P > 0.05) between CG and WZ samples during the entire 7-day postmortem storage period. Furthermore, our results showed that calpain-1 activation and autolysis were very extensive by day 1 in postmortem CG and WZ muscle, consistent with previous studies in chicken (Lee et al., 2008; Chang and Chou,



Figure 2. Zymograms showing postmortem changes in calpain-1, putative calpain-2, and calpain-11 activity of Chinese goose (A) and Wuzong goose (B) breast samples during postmortem storage at 5°C.



Figure 3. Postmortem changes in calpain-1, putative calpain-2 and calpain-11 in Chinese goose (A) and Wuzong goose (B) breast muscles stored at 5°C. The calpain-1, putative calpain-2 or calpain-11 activity is expressed as a percent of the total calpain activity (sum of the activities of calpain-1, putative calpain-2, and calpain-11) in the 0-day WZ (n = 15) samples, which was taken as 100%. Each time point of calpain-1, putative calpain-2 or calpain-11 activity is the average of CG (n = 15) or WZ (n = 15) samples. Bar = SE. Means between breed<sup>x, y</sup> or within a calpain isoform<sup>a-c</sup> without a common superscript differ (P < 0.05).

2010) and in duck (Liao and Chou, 2014; Liao et al., 2016) muscles.

Figure 3A also shows that the calpain-11 activity in 0-day CG samples (80% of total calpain activity) decreased slowly to 73%, to 66%, and to 64% of the total activity in 1-day, 3-day, and 7-day samples, respectively. Similarly, the 0-day WZ calpain-11 activity (77% of total calpain activity) decreased to 71%, to 56%, and to 37% of the total activity in 1-day, 3-day, and 7-day samples, respectively (Figure 3B). However, the calpain-11 activity in 7-day samples was significantly lower (P < 0.05) in WZ than in CG samples. The calpain-11 activity decreased more rapidly in WZ (loss 19%) than in CG (loss 2%) samples from day 3 to day 7 postmortem, suggesting a potential effect of goose breed on postmortem calpain-11 activation and autolysis.

Collectively, our results indicate that calpain-1 was activated and autolyzed within 1 D postmortem in both CG and WZ goose muscle. However, activation and autolysis of calpain-11 occurred not only later postmortem (3 to 7 D postmortem) in both CG and WZ goose muscle, but more rapidly in WZ than in CG muscles. Therefore, it could be assumed that



Figure 4. Western blots showing changes in desmin of Chinese goose (A) and Wuzong goose (B) breast muscle samples during postmortem storage at 5°C. Postmortem changes (C) in desmin content in Chinese goose ( $\blacksquare$ ) and Wuzong goose ( $\square$ ) breast muscles stored at 5°C. The desmin content is expressed as a percent of the desmin content in the 0-day (n = 15) WZ breast samples, which was taken as 100%. Each time point of desmin content is the average of CG (n = 15) or WZ (n = 15) samples. Bar = SE.

first capain-1 and later calpain-11 were involved in the postmortem proteolysis and tenderization of goose muscle.

## Postmortem Desmin Degradation

Postmortem desmin degradation is known to be highly associated with meat tenderization (Starkey et al., 2016). Immunoblotting results show that desmin bands (labeled as D) decreased in both CG (Figure 4A) and WZ (Figure 4B) samples with time postmortem. When desmin content in 0-day WZ samples was taken as 100%, the desmin content decreased form 0-day samples (97% CG vs. 100% WZ) to 1-day samples (91% CG vs. 87% WZ) and to 3-day samples (74% CG)vs. 77% WZ) and was not different in each respective day between CG and WZ samples. The desmin content decreased most rapidly from day 3 to day 7 and was lower (P < 0.05) in 7-day WZ (7%) than CG (24%) samples. Due to a similar rate of calpain-1 activation found in both breed muscles during the entire 7-day postmortem period (Figure 3), this difference in desmin content might be caused by a more rapid activation of calpain-11 in WZ than in CG muscle.

Table 1. Postmortem changes in shear force values (kg) in Chinese geese (CG) and Wuzong geese (WZ) breast samples stored at  $5^{\circ}$ C.

	Postmortem time, D	CG	WZ
Shear force	1 7	$\begin{array}{rrr} 10.5 \ \pm \ 0.2^{\rm a,x,*} \\ 8.2 \ \pm \ 0.1^{\rm b,x} \end{array}$	$\begin{array}{r} 10.3 \ \pm \ 0.2^{\rm a,x} \\ 7.7 \ \pm \ 0.1^{\rm b,y} \end{array}$

\*Within a row<sup>x, y</sup> or a column<sup>a, b</sup>, means without a common superscript differ in the measurement (P < 0.05).

# Postmortem Changes in Warner–Bratzler Shear Force Values

Table 1 shows that shear force values (kg) were (P < 0.05) lower in 7-day CG  $(8.2 \pm 0.2)$  and WZ  $(7.7 \pm 0.1)$  than in 1-day CG  $(10.5 \pm 0.2)$  and WZ  $(10.3 \pm 0.2)$  samples, respectively, in agreement with previous studies in goose muscle (Damaziak et al., 2016). Although 1-day shear force values were not different (P > 0.05) between CG and WG samples, the 7-day value was significant (P < 0.05) lower in WZ than in CG samples. These results are consistent with more extensive desmin degradation in WZ samples

(Figure 4C) during 7 D of postmortem storage and indicated that breed of goose could have an impact on postmortem meat tenderness. Result from other laboratories (Pringle et al., 1997; Kuber et al., 2004; Monsón et al., 2005; Marino et al., 2013) have also shown that beef breed can affect meat tenderness.

Our previous studies (Liao and Chou, 2014) showed that meat tenderness variation between Pekin and Muscovy duck muscles was due to a more rapid calpain-1 activation occurred in postmortem Pekin breast muscles. Present studies showed that although calpain-1 activity was not different between CG and WZ muscles (Figure 2), calpain-11 activity (Figure 3) was activated more rapidly in WZ than in CG postmortem muscles. These results might, at least, partially explain the meat tenderness variation between 2 goose muscles. On the other hand, previous studies showed that the tenderness variation between Waguli and Brahman muscles might be attributed to a higher level of calpastatin, a calpain-specific inhibitor, and activity present in Brahman muscle (Ibrahim et al., 2008). Accordingly, this might imply that the calpastatin activity was also a factor partly affecting the tenderness variation observed between CG and WZ muscles. However, it is not known whether the difference in gene characteristics between those 2 breeds could affect the calpastatin activity in goose muscle. Therefore, further studies of the mRNA expression of calpastatin in CG and WZ goose muscles would be needed.

## CONCLUSION

Our results show that the decline in pH and calpain-1 activity were similar in CG and WG samples. However, the postmortem decrease in calpain-11 activities, desmin content and shear force was more rapid (P < 0.05) in WZ than in CG samples, implying that not only calpain-1 but also calpain-11 was involved in proteolysis and tenderization of postmortem goose muscle. Therefore, these results show that postmortem proteolysis and tenderization of goose breast muscle were more extensive in WZ than in CG goose muscle. Present study suggests that this variation in meat tenderness between goose breeds might be a factor required for further processing considerations in goose industry.

### CONFLICT OF INTEREST

There is no conflict of interest.

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