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Research Article
Comparison of Levels of Calpains and Calpastatin in Blood and their Distribution in Skeletal Muscle of Turkey

L. Kripriyalini, Ashim K. Biswas, Samarth Tandon, C.K. Beura and A.S. Yadav

Division of Post-Harvest Technology, ICAR-Central Avian Research Institute, Izatnagar, 243122 Bareilly, Uttar Pardesh, India

Abstract

Background and Objective: Tenderness is one of the most important quality parameters significantly influencing the eating quality of meat. So, the aim of the study was to compare the concentration of calpains and calpastatin enzymes in blood and their distribution in skeletal muscles of turkey to predict possible role of these enzyme in tenderization of meat when the turkey is still alive or waiting for slaughter. **Materials and Methods:** Calpains and calpastatin enzymes were extracted from blood and skeletal muscle samples using tris-buffer of 50 mM (pH 6.7), dialyzed at 12 kDa MWCO cellulose filter and finally purified and separated on DEAE-Sephacel anion exchange column. Activity analysis was performed using spectrophotometric technique. **Results:** Study revealed that breast muscle contained significantly ($p<0.05$) higher μ-calpain concentration as compared to blood and thigh muscles. But m-calpain concentration was higher in thigh muscle than breast or >blood. Concentrations of both the domains of caplains (μ and m) were also differed significantly ($p<0.05$) in blood, breast and thigh muscle samples in between 42 and 32 weeks age groups of male and female turkey. Significant ($p<0.05$) differences were also found in calpastatin levels amongst the samples. **Conclusion:** The study envisaged that breast muscle contained highest amount m-calpain but intermediate concentration of μ-calpain and calpastatin those were higher than the concentration determined from blood samples. This study confirmed the importance of determining both the calpains as well as calpastatin in blood since values generated from this sample has delivering direct information for possible concentration of these enzymes may found in breast and thigh muscles.

Key words: μ-calpain, m-calpain, calpastatin, turkey meat, tenderization, blood, breast muscle, CASF, tendering enzyme

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Corresponding Author: Ashim K. Biswas, ICAR-Central Avian Research Institute, Izatnagar, 243122 Bareilly, Uttar Pardesh, India Tel: +91-581-2303223 Fax: +9-581-2301321

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Calpains (μ and m), a calcium-dependent protease has been recognized as key player in post-mortem tenderization of skeletal muscle. Calpastatin, another endogenous enzyme of calpain system is inhibitory to activity calpains and is widely distributed in muscle tissues. In poultry and in particularly in turkey breast muscle, the role of post-mortem proteolysis is poorly documented and few studies performed did not take into account the particularities of the calcium-dependent proteases in these species[1]. It was recently showed that the distribution of the calpains in turkey varies from one tissue to another[2]. In *P. superficialis* muscle and iliotibialis muscle, the ratio of μ-calpain to μ/m-calpain is 1:10, so there is a marked predominance of μ/m-calpain[2]. The enzymes are also present in blood, however, their concentration is variable depending on species, breed, sex, age, etc. and their determination in it predict post-mortem tenderization of meat. But enzymatic concentration in blood may not be the same as distributed in muscle tissues for their activity due to biological variations such as pH, ionic strength, temperature etc. In fact, by determining the enzymatic concentrations in live animals and in post-mortem muscle, it can be managed tenderness of meat to maximize consumer quality perception. Therefore, quantification of calpastatin, μ- and m-calpains in tissue system is frequently, an essential element of studies aimed at explaining variation in tenderness of turkey meat from different age, sex or muscles. It is generally accepted that the process of meat tenderization is essentially enzymatic and is dependent on the physicochemical conditions such as pH and ionic strength. Proteolysis of myofibrillar and its associated proteins tenderizes the meat by weakening intra and inter-myofibrillar bonds[3,4]. As calpains are also present in turkey muscles[5] they may have a similar role in the tenderization process.

Many studies were conducted to extract and separate μ- and m-calpains as well as calpastatin to assess their possible role in post-mortem tenderization of various muscle tissues from other meat animals[6,7]. Since limited studies were carried out to compare the concentration of calpains and calpastatin enzymes in turkey blood and their distribution in skeletal muscles, this study is aimed to determine calpains and calpastatin concentration in them to predict their possible role in post-mortem tenderization of turkey meat.

MATERIALS AND METHODS

Collection of samples: A total 12 turkey birds of both sexes comprised of 3 birds from each of 42 and 32 weeks of age groups were selected from Experimental Turkey Farm of ICAR-CARI, Izatnagar, Bareilly. A total of 24 tissue samples comprised each of 12 breast and thigh muscles were collected from Experimental Poultry Processing Plant of Division of Post-Harvest Technology, ICAR-Central Avian Research Institute, India. The birds were slaughtered as per standard slaughtering practices. Immediately after exsanguination, the skin covering breast and thigh muscles were removed and excised. About 100 g of meat samples were collected from grading table and transferred in to a self-sealing LDPE bags until analysis.

Blood samples (25 mL each of 12 birds) were collected in vials containing EDTA (1 mg mL^{-1} blood) during bleeding operations where they were hung, stunned and slaughtered manually. Then, it was transferred to the laboratory for processing immediately.

Preparation and extraction of samples: The procedure followed for extraction of μ- and m-calpains and calpastatin was a modification of the method described by Koohmaraie[8]. Briefly, freshly collected tissue samples were cut in to fine pieces after being trimmed of excessive connective tissues, fat and fascia. About 0.5 g of finely cut muscle samples were homogenized with 6 volumes of ice-cooled extraction buffer containing 50 mM tris-base (pH 6.7) along with 10 mM EDTA and 0.05% (v/v) 2-mercaptoethanol (MCE). To avoid the functioning of unwanted enzymes, protease inhibitors [2 mM phenylmethane sulfonylfluoride (PMSF), 100 mg L^{-1} ovomucoid, 6 mg L^{-1} leupeptin] were added in the extraction buffer just before preparation of meat homogenate. Extracts were centrifuged at 12000 rpm for 20 min at 4°C (Make-Eppendorf 5427R, Germany). Supernatants were collected in separate centrifuge tube and sediments were disposed-off. The collected supernatant was centrifuged once again as mentioned earlier and was later filtered[9-11]. Similarly for blood samples (1.15 mL), 3 volumes of extraction buffer (as mentioned earlier) were used but additionally 0.1% triton X-100 (v/v) was incorporated, while making the blood homogenate. Other processing steps were same as mentioned for meat homogenate.

Purification and separation of μ- and m-calpains and calpastatin from sample extract: For purification, dialysis was performed using dialysis tubing of 12 kDa MWCO cellulose filter (Sigma-Aldrich, USA) in which supernatant obtained after extraction was kept overnight at 4°C in dialysis buffer (pH 7.4) containing 40 mM tris-base, 5 mM EDTA and 0.05% (v/v) MCE in ratio of 20:1 of buffer and sample and was latter centrifuged at 7,000 rpm for 10 min at 4°C.

Supernatant was collected, filtered through a Whattman filter paper No. 1 and loaded on a pre-conditioned econo-column (W×L, 1.5×8.5 cm) supplied by Bio-rad Laboratories, Lucknow, India. Swollen DEAE-Sephacel (Sigma-Aldrich, USA) was used as column matrix. DEAE-Sepharose column was equilibrated with equilibration buffer (pH 7.4) comprising of 40 mM tris-base, 0.5 mM EDTA and 0.05% (v/v) MCE. After 3 times washing with equilibration buffer (3×20 mL), sample extract corresponding to 0.5 g of meat or 1.15 mL of blood sample was loaded on a column having 5 cm settled DEAE-Sephacel matrix which was standardized after minor modification from the previous studies[12]. The elution was carried out in a step-wise increase of NaCl concentrations. Fractions of 2 mL were collected from each NaCl gradient. Calpastatin and m-calpain were eluted from the columns using a 100 and 400 mM NaCl gradient, whereas μ-calpain was eluted with 200 mM NaCl. Fractions were stored at 4°C until assayed.

Calpain and calpastatin assays: Enzymatic activities of calpains (μ and m) and calpastatin were determined using casein as a substrate as described by Dayton *et al.*[13] with slight modification. For calpain activity determination two types of assay buffer were used. The fractions containing μ- and m-calpains were pooled separately. An aliquot of 0.5 mL from each fraction with potential activity was screened for activity by mixing with 1.5 mL of assay buffer containing 100 mM tris-base (pH adjusted at 7.5 with 1 N acetic acid), 5 mM $CaCl_2$, 1 mM NaN_3, 5 mg mL^{-1} casein and 10 mM 2-MCE. The reactions were incubated for 60 min at 25°C and stopped by adding 2 mL of a 5% trichloroacetic acid solution (TCA). The denatured proteins were precipitated by centrifugation at 2000 rpm for 30 min and the soluble peptides were measured for absorbance at 278 nm using Biospectrometer, Eppendorf, Germany. For determination of calcium-independent proteolytic activity of each fraction, $CaCl_2$ in the reaction mixture was replaced by 10 mM EDTA. To determine Ca^{2+}-dependent proteolytic activity, a bsorbance at A_{278} in the presence of EDTA was subtracted from that of the $CaCl_2$ reactions. Blanks were made using 0.5 mL of 200 and 400 mM NaCl in equilibration buffer for μ- and m-calpains containing fractions respectively in 1.5 mL of assay buffer containing either $CaCl_2$ or EDTA. Total activity was calculated by multiplying Ca^{2+}-dependent proteolytic activity by the dilution factor:

$$\text{CDP activity (U g}^{-1}) = (A_{278}\ CaCl_2\ \text{buffer-}A_{278}\ \text{EDTA buffer})\times\text{dilution factor}$$

One unit of calpain activity was defined as the amount of enzyme that catalyzed an increase of 1.0 absorbance unit at A_{278} after 60 min at 25°C.

For calpastatin activity, fractions potentially containing calpastatin and m-calpain at 4°C were pooled for 1 min before adding 1.5 mL assay buffer containing $CaCl_2$ to start the reaction. The reaction was stopped after 60 min with 2 mL of 5% TCA and then centrifuged for 30 min and absorbance at 278 nm was measured as before. Three tubes were used to assay inhibitor activity: (1) m-calpain pooled fraction, incubated with assay buffer containing $CaCl_2$, (2) m-calpain fraction plus calpastatin fraction, incubated in assay buffer as described in 1, (3) Calpastatin fraction alone, incubated in assay buffer containing EDTA. Total inhibitor activity was calculated according to the equation:

$$\text{Total inhibitor activity (U g}^{-1}) = 1\text{-}(2\text{-}3)\times\text{dilution factors}$$

One unit of calpastatin activity was defined as the amount of calpastatin that inhibits one unit of m-calpain[8,14].

Determination pH: The pH of different breast and thigh muscles were determined immediately after slaughter as per the methodology mentioned by Trout *et al.*[15] to study its influence on calpains and calpastatin activity during post-mortem. The pH value was measured with a Bench top digital pH meter (Eutech 2700) equipped with glass electrode and automatic temperature sensors on 10 g of sample homogenized with 50 mL of distilled water using pestle and mortar for 2 min. The pH values were recorder and changes of activity of μ- and m-calpains and calpastatin were observed.

Determination of W-B shear force value: Warner-Bratzler shear blade attached to TA-HDi Texture Analyser (Stable Micro System, UK) equipped with 50 kg load cell was used to evaluate the effect of aging on tenderness. Test speed was 4 mm sec^{-1} and travel distance was 25 mm. Maximum force required to cut strips was measured as kilograms.

Statistical analysis: Experimental data generated were analysed statistically using standard software package as mentioned by Snedecor and Cochran[16]. Means of calpains (μ and m) and calpastatin activity obtained for comparison of male and female were analysed using independent paired t-test. Other data relating to overall means were evaluated two-way ANOVA, homogeneity test and paired t-test for comparing means to find the effects between breast and

thigh muscles, between male and female and their interactions. The statistical significance was expressed at $p<0.05$.

RESULTS AND DISCUSSION

Comparison of enzymes concentration between male and female: Results of μ- and m-calpains and calpastatin concentrations for 32 and 42 weeks of both the male and female turkey are showed in Table 1-3. It has been observed that levels of enzymes in between the male and female birds of each age group were differed non-significantly ($p>0.05$). However, μ-calpain concentration was significantly ($p<0.05$) higher in breast muscles as compared to blood and thigh muscles. Similar results were observed for 42 weeks within blood, breast and thigh. The male and female of 32 weeks as well as for 42 weeks showed significant ($p<0.05$) differences within blood, breast and thigh for m-calpain. The thigh muscle was significantly higher when compared to breast muscles then followed by blood. Similarly, in case of calpastatin thigh muscle was significantly higher than breast muscle and blood. As expected significant difference ($p<0.05$) of μ-calpain concentrations were observed in between 42 and 32 weeks age group and with the higher levels in breast muscles as compared to blood and thigh muscles irrespective different age and sex of turkey. The overall mean of μ-calpain concentrations were followed as M42>F42 but differed non-significantly. Similar observations also were found when compare concentrations in between 32 weeks age group of male and female turkey (M32 and F32).

Since, there are no other published literature on the comparative studies for concentration of μ-calpain in blood and skeletal muscle tissues of turkey, the data reported here in this study cannot be directly and quantitatively compared due to significant and variable changes in activity by these enzyme that convened immediately after slaughter and subsequent processing. However, the lower concentration of this enzyme in thigh muscles in all groups and sexes of turkey could be due to genetic variations coupled with rapid autolysis and comparatively lower level of pH. Activity of μ-calpain is also highly muscle specific. On the other hand, μ-calpain concentration was highest in breast muscle due to different muscle type and existence of favourable pH that required for activation. The intermediate activity by μ-calpain was found in blood sample. However, significant variations of μ-calpain activity amongst the different age groups and sexes of turkey could be correlated due to genetic variations and quality of proteomes at different stages of growth.

Table 1: Concentration of μ-calpain in blood, breast and thigh muscles of 32 weeks age group between male and female of turkey

Treatments	Blood	Breast	Thigh	Overall treat Mean±SE
μ-calpain				
M32	0.18 ± 0.04^{b}	0.29 ± 0.04^{c}	0.07 ± 0.01^{a}	0.18 ± 0.04
F32	0.27 ± 0.04^{b}	0.23 ± 0.04^{b}	0.06 ± 0.02^{a}	0.19 ± 0.03
Overall Mean±SE	0.22 ± 0.04^{b}	0.26 ± 0.04^{c}	0.07 ± 0.01^{a}	
m-calpain				
M32	0.85 ± 0.04^{a}	2.46 ± 0.05^{bAB}	2.75 ± 0.05^{cAB}	2.02 ± 0.05
F32	0.89 ± 0.04^{a}	2.37 ± 0.04^{bA}	2.66 ± 0.04^{cA}	1.97 ± 0.04
Overall Mean±SE	0.87 ± 0.04^{a}	2.41 ± 0.05^{b}	2.71 ± 0.05^{c}	
Calpastatin				
M32	0.18 ± 0.01^{aA}	0.55 ± 0.06^{b}	0.68 ± 0.04^{b}	0.47 ± 0.03
F32	0.20 ± 0.03^{aAB}	0.51 ± 0.05^{b}	0.65 ± 0.06^{b}	0.45 ± 0.04
Overall Mean±SE	0.19 ± 0.02^{a}	0.53 ± 0.05^{b}	0.67 ± 0.05^{b}	

Means bearing different superscript row-wise (small letter) differ significantly ($p<0.05$). M32: 32 weeks male, F32: 32 weeks female

Table 2: Concentration of μ-calpain in blood, breast and thigh muscles of 42 weeks age group between male and female of turkey

Treatments	Blood	Breast	Thigh	Overall treat Mean±SE
μ-calpain				
M42	0.25 ± 0.03^{b}	0.55 ± 0.04^{c}	0.08 ± 0.03^{a}	0.29 ± 0.03
F42	0.34 ± 0.05^{b}	0.53 ± 0.03^{c}	0.07 ± 0.02^{a}	0.31 ± 0.04
Overall Mean±SE	0.30 ± 0.05^{b}	0.54 ± 0.04^{c}	0.08 ± 0.03^{a}	
m-calpain				
M42	0.93 ± 0.04^{a}	2.53 ± 0.04^{b}	2.94 ± 0.04^{c}	2.13 ± 0.04
F42	1.02 ± 0.04^{a}	2.47 ± 0.05^{b}	2.88 ± 0.05^{c}	2.12 ± 0.05
Overall Mean±SE	0.97 ± 0.04^{a}	2.50 ± 0.04^{a}	2.91 ± 0.04	
Calpastatin				
M42	0.19 ± 0.02^{a}	0.74 ± 0.05^{b}	0.82 ± 0.06^{b}	0.58 ± 0.04
F42	0.28 ± 0.03^{a}	0.67 ± 0.05^{b}	0.76 ± 0.07^{b}	0.57 ± 0.05
Overall Mean±SE	0.24 ± 0.03^{a}	0.71 ± 0.05^{b}	0.79 ± 0.07^{b}	

Means bearing different superscript row-wise (small letter) differ significantly ($p<0.05$). M42: 42 weeks male, F42: 42 weeks female

Table 3: Concentration of μ-calpain in blood, breast and thigh muscles of different age group and sex of turkey

Treatments	Blood	Breast	Thigh	Overall treat Mean±SE
μ-calpain				
M32	0.18 ± 0.04^{bA}	0.29 ± 0.04^{cA}	0.07 ± 0.01^{a}	0.18 ± 0.04^{A}
F32	0.27 ± 0.04^{bAB}	0.23 ± 0.04^{bA}	0.06 ± 0.02^{a}	0.19 ± 0.03^{A}
M42	0.25 ± 0.03^{bAB}	0.55 ± 0.04^{cB}	0.08 ± 0.03^{a}	0.29 ± 0.03^{B}
F42	0.34 ± 0.05^{bB}	0.53 ± 0.03^{cB}	0.07 ± 0.02^{a}	0.31 ± 0.04^{B}
Overall Mean±SE	0.26 ± 0.04^{b}	0.40 ± 0.03^{c}	0.07 ± 0.02^{a}	
m-calpain				
M32	0.85 ± 0.04^{aA}	2.46 ± 0.05^{bAB}	2.75 ± 0.05^{cAB}	2.02 ± 0.05^{B}
F32	0.89 ± 0.04^{aAB}	2.37 ± 0.04^{bA}	2.66 ± 0.04^{cA}	1.97 ± 0.04^{B}
M42	0.93 ± 0.04^{aAB}	2.53 ± 0.04^{bB}	2.94 ± 0.04^{cC}	2.13 ± 0.04^{A}
F42	1.02 ± 0.04^{aB}	2.47 ± 0.05^{bAB}	2.88 ± 0.05^{cBC}	2.12 ± 0.05^{A}
Overall Mean±SE	0.92 ± 0.04^{a}	2.46 ± 0.05^{b}	2.81 ± 0.04^{c}	
Calpastatin				
M32	0.18 ± 0.01^{aA}	0.55 ± 0.06^{bA}	0.68 ± 0.04^{b}	0.47 ± 0.03^{A}
F32	0.20 ± 0.03^{aAB}	0.51 ± 0.05^{bA}	0.65 ± 0.06^{b}	0.45 ± 0.04^{A}
M42	0.19 ± 0.02^{aAB}	0.74 ± 0.05^{bB}	0.82 ± 0.06^{b}	0.58 ± 0.04^{B}
F42	0.28 ± 0.03^{aB}	0.67 ± 0.05^{bAB}	0.76 ± 0.07^{b}	0.57 ± 0.05^{B}
Overall Mean±SE	0.21 ± 0.03^{a}	0.62 ± 0.05^{b}	0.72 ± 0.06^{c}	

Means bearing different superscript row-wise (small letter) and column-wise (capital letter) differ significantly ($p<0.05$). M32: 32 weeks male, F32: 32 weeks female, M42: 42 weeks male, F42: 42 weeks female

In comparison to μ-calpain, m-calpain so called calpain II is more stable at wider range of pH and its concentration may found up to 10 times to that of μ-calpain. For this, concentration of m-calpain was found significantly ($p<0.05$) higher than μ-calpain in different blood and muscle samples. Amongst two different muscle samples, thigh sample of 42 weeks age group of female turkey contained higher m-calpain than either 32 or 42 weeks male/female thigh and breast samples. These findings are agreed with results reported by Lee *et al.*[2] in chicken muscles and according to them the distribution of μ- and m-calpains in chicken varies from one tissue to another. In breast muscle and thigh muscle, the ratio of μ-calpain to m-calpain is 1:10 i.e., there is a marked predominance of m-calpain. They also again demonstrated that chicken calpains are more calcium-sensitive than mammalian calpains and may therefore, play a special role in the post-mortem process in chicken muscle, explaining its rapidity. The concentration of calpains in blood and in parallel measured the activity in muscle samples and it was found there was a strong correlation of these enzymatic concentrations in blood and other muscle tissues which in turn helps in assuming post-mortem proteolysis of turkey muscle.

The highest calpastatin concentration was observed in thigh muscles followed by breast and lowest in blood samples among all the age groups and sex of turkey. The calpastatin concentration of thigh muscles was significantly ($p<0.05$) higher than blood but that was differed non-significantly with breast muscles in all the age and sex. For blood samples, calpastatin concentration was observed to be highest in 42 weeks age group of female turkey (F42) and it was significantly higher than M32. In case of breast muscle, similar observations of calpastatin concentration were found in between 32 weeks age of male (M32) and 42 weeks of female (F42) but non-significant differences were found in calpastatin concentration when it was compared within the same age group and sex of turkey.

The calpastatin though do not have direct role in post-mortem proteolysis but it indirectly influence the proteolytic activity by inhibiting activity of calpains. As expected highest level of calpastatin was observed in thigh muscle which could be due to biological variation and location of the muscle[2]. Blood samples showed very little calpastatin which could due to rapid degradation of this enzymes as that was found for μ-calpain. Indeed, significant variations were observed in calpastatin concentration amongst the different muscles irrespective of age and sex. In a study with turkey muscle similar findings were reported by Obanor *et al.*[1]. These researchers reported that calpain I (μ-calpain) and calpastatin activities declined very rapidly over the first 3 h and were no longer detected 24 h post-mortem. In this study calpastatin was remained undetectable in blood sample.

Table 4: Changes in pH and Warner-Bratzler shear force value of turkey breast and thigh muscles immediately after processing

Treatments (Birds)		pH	W-B shear force (kg cm^{-2})
32 weeks	MBM	6.65±0.10	5.67 ± 0.03^{D}
	MTM	6.57±0.07	5.55 ± 0.03^{C}
	FBM	6.85±0.07	4.07 ± 0.04^{B}
	FTM	6.75±0.21	3.76 ± 0.02^{A}
Overall		6.71±0.11	4.76±0.03
42 weeks	MBM	6.64±0.05	6.61 ± 0.02^{D}
	MTM	6.57±0.03	6.37 ± 0.03^{C}
	FBM	6.80±0.04	5.18 ± 0.20^{B}
	FTM	6.69±0.03	4.51 ± 0.03^{A}
Overall		6.68±0.04	5.67±0.03

Means bearing different superscript column-wise (capital letter) differ significantly ($p<0.05$). M32: 32 weeks male, F32: 32 weeks female, M42: 42 weeks male, F42: 42 weeks female

Effect of pH: The results of pH value of turkey muscle samples immediately after slaughter are presented in Table 4. There was non- significant ($p>0.05$) difference in pH value amongst the treatments. The rate of pH fall depends on the initial concentration of glycogen, the rate of glycolysis, animal species, muscle types, pre-slaughter treatment and storage temperature[17]. It has been reported that the post-mortem pH decline is linked to glycogen depletion with the pH of turkey or chicken breast muscle declining more rapidly than lamb or beef[18]. The result found in this study is consistent with previous studies that show the breast muscle of turkey exhibits accelerated rigor mortis compared with beef, lamb or chicken muscles[2,19,20]. As calpains are also present in turkey muscles[5] they may have a similar role in the tenderization process in these species.

Effect on W-B shear force value: The W-B shear force value of different types of birds of same age group was differed significantly ($p<0.05$) in following orders MBM>MTM>FBM>FTM (Table 4). Similar findings were also observed for 42 weeks of age, however, higher W-B shear force value was recorded in 42 weeks when compared to 32 weeks of age group of turkey. Further, immediately after slaughtering of turkey the shear-force value was maximum for breast muscle than thigh. This indicates that breast meat was less tender at death than thigh meat. Higher shear force value for the muscles from 42 weeks age group of turkey could be attributed to age related factors due production of more intermolecular collagen linkages. But variation in shear force value in between male and female birds could be due to variation in rate of muscle development. Similar findings were also reported by Liu *et al.*[21].

CONCLUSION

In general, breast muscle contained highest amount of μ-calpain but intermediate concentration of m-calpain and calpastatin. This study also confirmed the importance of determining both the calpains as well as calpastatin in blood, since values generated from this sample has delivering direct information for concentration of these enzymes present in breast and thigh muscle.

SIGNIFICANCE STATEMENT

By determining calpains and calpastatin concentration in blood, it may possible to predict concentration of these enzymes in post-mortem muscle and their possible role in post-mortem tenderization of meat.

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