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Biochemical Basis of Calpain and Calpastatin Activity Determination in Blood and Tissue Samples: Potential Role in Post-Mortem Ageing of Guinea Fowl Meat

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Abstract

Calpains, the calcium-dependent thiol-proteases have been implicated in various important cellular processes including tenderization of meat. So, aim of this study was to identify calpains and their potential inhibitor calpastatin in blood and skeletal muscles to elucidate their role in post-mortem tenderization of guinea fowl meat during holding at 4±1 °C. For this, breast and thigh muscles of two different guinea fowl varieties were collected, processed, and finally, analyzed on casein Zymogram gels. The sample extracts were also subjected to dialysis (12 kDa MWCO) and loaded on DEAE anion column for purification and separation of μ- and m-calpains as well as calpastatin. Results show that there were clear bands of μ- and m-calpains in the extracts of both the blood and muscle samples. But the band intensity for muscle samples kept decreasing with the increase of holding time showing the decreasing trend in activity of these enzymes. The calpastatin activity was also decreased rapidly and greatly. The pH and W-B shear force values were decreased with the increase in holding time. Finally, the μ-calpain induced post-mortem maturation time was optimized at 6 h for breast and thigh muscles of both the varieties of guinea fowl.

Keywords: Calpains; Zymography; Ageing; Guinea Fowl Meat; Chromatography

Introduction

Guinea fowl (Numida meleagris) meat is a nutritionally desirable alternative to consumers than meat from other livestock and poultry species [1]. During the past few years much of the poultry consuming countries have switched to this bird to produce meat for luxury markets, and there is still a vast, untapped future for its meat [2]. Guinea fowl meat is preferred on account of its dark gamey taste, colour and higher mineral composition but

in low in fat content than other poultry species. The meat from this species could be an excellent and healthy alternative to the consumers, but only little research exploitation has been done for improvement its quality, in particularly tenderness. In comparison to broiler meat and also other chicken varieties, meat from guinea fowl is tougher even at its slaughter weight at 12 weeks of age. It has been well documented that post-mortem acidification/proteolysis of meat has greater role in improvement of tenderness [2, 3, 4, 5, 6] but this phenomenon of post-mortem acidification is also poorly documented for this species [7]. Earlier studies demonstrated that calpain system comprising of calcium-dependent endogenous proteases μ-calpain, m-calpain and their inhibitory enzyme calpastatin have a major role in post-mortem skeletal muscle breakdown which results in tenderness of meat [8]. Moreover, the role of these enzymes in blood still requires meticulous studies, due to variation in the two biological matrices.

Although the process of meat tenderization during post-mortem holding of carcasses is a well-known practice, the mechanism through which these changes are brought remains elusive and controversial.

However, the calpain theory of tenderization was recognized as the most probable theory for post-mortem changes of muscles [9]. Under this theory it is also well documented that μ-calpain and calpastatin progressively lost their activity during post-mortem maturation, but m-calpain remained stable. So, to better understand the role of calpain system in meat tenderness, this study contemplates to identify the activity of μ- and m-calpains and calpastatin in blood and tissue samples from two different varieties, and also to understand their possible role in post-mortem tenderization of breast and thigh muscle during holding at 4±1 °C. The changes in muscle pH, W-B shear force value were also monitored to optimize μ-calpain induced post-mortem maturation of guinea fowl meat from two different varieties.

Materials and methods

Chemicals and Reagents

Casein from bovine milk (purified powder); dialysis tubing cellulose membrane (12 kDa-MWCO), DEAE-Sephacel (anion exchanger), protease inhibitors (leupeptin hemisulfate, ovomucoid) were procured from Sigma-Aldrich, USA. Another protease inhibitor phenylmethane sulfonylfluoride (PMSF) was obtained from Sisco Research Laboratories, Mumbai, India. Novagen perfect protein marker (10-225 kDa) was obtained from Merck Millipore, Mumbai, India. An Econo-Column (1.5 x 8.5 cm) used for chromatography analysis was procured from Bio-Rad Laboratories, Lucknow, India. All other solvents and reagents required for this experiment were of standard grade and procured from s. D. Fine Chemicals, New Delhi, Sisco Research Laboratories, Mumbai and Merck Specialist Pvt. Ltd., Mumbai, India.

Raw Material Collection

A total of 80 muscle samples (comprising 40 breast and thigh muscles) from 80 culled (52 wk age) guinea fowl birds (40 Pearl and 40 Swetambari) were collected from Experimental Poultry Processing Plant of ICAR-Central Avian Research Institute, India. The birds were slaughtered as per standard slaughtering practices. Immediately after exsanguination, the skin covering muscles was opened and the breast and thigh muscles were collected. The samples were collected from grading table where tissue samples were excised, packed and chilled immediately. About 300 g of muscle samples were collected and transferred in to a self-sealing LDPE bags. The bags were labeled and transported to laboratory under chilled condition for analysis. Blood samples (15 mL from each bird) were collected from the birds during bleeding operations where they were hung, stunned and slaughtered manually. The blood samples were collected in the vials containing EDTA (1 mg/mL blood) and transferred to the laboratory for processing immediately.

Processing and extraction of samples

For processing of samples, excessive connective tissues, fat and fascia were trimmed-off and each muscle sample was sub-divided into three individual groups. All samples were held at 4±1 ºC for evaluation of biochemical changes at 0, 6 and 24 h of post-mortem maturation. Accurately 3 g of these finely cut sample was weighed and homogenized using pestle and mortar- in pre-chilled conditions with 6 volumes of ice-cooled extraction buffer comprising of tris-base 50 mM (w/v) (pH 8.3), 10 mM EDTA (w/v) and 0.05 % (v/v) 2-mercaptoethanol (MCE). Protease inhibitors [2 mM (PMSF), 100 mg/L ovomucoid and 6 mg/L leupeptin] were incorporated in the extraction buffer just before use to avoid the functioning of unwanted enzymes. The extract was then subjected to centrifugation (Make-Eppendorf 5427R, Germany) at 12000 rpm for 20 min at 4 °C. Supernatant was decanted in a separate centrifuge tube and sediment was disposed-off. The collected supernatant was centrifuged once again as mentioned earlier and was later filtered and collected

in a separate centrifuge tube [10]. Both breast and thigh samples from all the birds were processed similar manner at different time of PM ageing. Likewise, for blood, samples (1.15 mL) were homogenized with 3 volumes of extraction buffer (as prepared previously), but additionally 0.1% Triton X-100 (v/v) was included while making blood extracts. Other processing steps remained same as mentioned above for meat extract preparation.

Purification and Separation

Purification and separation steps are mandatory for proper identification of calpains and calpastatin. In order to achieve this, supernatant as obtained was first kept in a dialysis buffer (pH 7.4) comprising 40 mM tris-base, 5 mM EDTA and 0.05% (v/v) MCE. The ratio of buffer and sample extract was maintained at 20:1 or more. Dialysis was performed overnight at 4°C using dialysis tubing of 12 kDa MWCO cellulose filter (Sigma-Aldrich, USA). Fresh dialysis buffer was prepared for each sample and that was stored at 4±1 °C until use.

For purification and separation of μ-calpain, m-calpain and calpastatin, anion exchange chromatography was performed in which supernatant obtained after dialysis was loaded on a pre-conditioned Econo-Column (W×L; 1.5 x 8.5 cm) supplied by Bio-Rad Laboratories, Lucknow, India. Swollen DEAE-Sephacel (Sigma-Aldrich, USA) was used as column matrix. The column was equilibrated through three washings (3 × 20 mL) with equilibration buffer (pH 7.4) containing 40 mM tris-base, 0.5 mM EDTA and 0.05% MCE. Sample extract corresponding to 3 g of meat or 1.15 mL of blood sample was loaded on a column having 5 cm settled DEAE-Sephacel. The calpains and calpastatin were eluted using gradient elution method with increasing concentrations of NaCl solution. Calpastatin were eluted first at 100 mM NaCl followed by μ- and m-calpain at 200 mM and 400 mM NaCl concentrations, respectively [11].

Analysis

Casein Zymography

Casein zymography method was performed for the detection of calpains as per methodology of Huang [4] with suitable modification. In modified method, the casein gels were pre-run on Mini-PROTEAN tetra system (Bio-Rad Laboratories, USA) at 100 V for 15 min at 4 °C with running buffer (pH 8.3). Sample was prepared by mixing the crude extract with sample buffer (3:1, v/v), and was then loaded on the casein gel and subjected to electrophoresis at 100 V for 4 h at 4 °C. After completion of electrophoresis, the gels were incubated for 18-24 hrs at 20°C in proteolytic buffer (pH 7.4) comprising 20 mM tris-base, 10 mM Ditheothreitol (DTT) at CaCl2 concentration of 4 mM. The gel was then stained for 60 min with Coomassie Brilliant Blue G-250 and then kept overnight in de-staining solution which gives clear bands of calpains [12]. The presence of calpains was clearly identified due to the digestion of casein molecules into small fragments that diffuse out of the gel.

SDS-PAGE Analysis

As calpastatin along with its different domains lacks the proteolytic activity, casein zymography cannot be used for its detection. Considering this, SDS-PAGE analysis was performed for identification of calpains and calpastatin from the purified fractions obtained after anion exchange chromatography [11]. In this method, resolving gel was polymerized with 0.05% (w/v) APS and 0.05 % (v/v) TEMED while the stacking gel containing was polymerised with 0.05% APS and 0.1% TEMED. The purified fractions were diluted with sample buffer (1:2, v/v) and then heated at 95°C for 5 minutes. The samples were loaded in the gel and electrophoresis was performed at constant voltage of 120 V for 90 min with the electrode running buffer. On completion of electrophoresis the gel was stained for 1h in the solution containing 0.1% (w/v) Coomassie Brilliant Blue G-250, 40 % (v/v) methanol and 10% (v/v) acetic acid and was destained in a solution comprising 30 % (v/v) methanol and 10 % (v/v) acetic acid [13].

Determination of calpains and calpastatin activity

Calpain activity was determined based on the extent of proteolysis of calpains and by measuring the amount of peptides released from casein by calcium dependent proteases. The calpastatin activity was measured by incubating appropriate amounts of pooled fractions containing calpastatin and m-calpain at 4°C for 1 min before adding 1.5 mL assay buffer containing CaCl2 to start the reaction. The reaction was stopped by adding 5 % TCA and centrifuged as mentioned earlier. Three tubes were used to assay inhibitor activity: (1) m-calpain pooled fraction, incubated with assay buffer containing CaCl2 ; (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 formula: Total inhibitor activity (Units/g) = 1 - (2 - 3) × dilution factors [14,15].

Determination pH

The pH of muscles was determined at various time intervals (0, 6, 24 hrs) as per the standard method to study its influence on calpains and calpastatin activity during post-mortem maturation at 4±1 °C [16]. 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 correlated with changes in activity of μ- and m-calpains and calpastatin.

Determination 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 maturation on tenderness and correlate the results with zymography and SDS-PAGE analysis. Test speed was 4 mm/s and travel distance was 25 mm. Maximum force required to cut raw strips (3cm ×1cm ×1 cm; L×W×H) was measured as Kg/cm2.

Statistical analysis

Experimental data generated were analyzed statistically using standard software package [17]. Means of calpains and calpastatin activity and other data of physicochemical analysis relating to post-mortem maturation were evaluated using two-way ANOVA. Homogeneity test and DMRT were used for comparing means to find the effects between post-mortem times and their interactions were performed. The statistical significance was expressed at $P < 0.05$.

Results and discussion

Casein zymography

Casein zymography method is based on the principle that casein molecules present in the gel are catalyzed by calpains in the presence of Ca^{2+} ion in solution. Both blood and muscle sample extracts showed ample proteolytic activity of calpains on zymography gel. However, the proteolytic activity of extracts obtained from muscle samples showed the decreasing trends with the increase in post-mortem maturation at 4 ± 1 °C. Among the two varieties the band intensity for Swetambari was greater than that identified for Pearl. Similar results were obtained for blood samples. It has also been found that the bands obtained from breast muscle samples showed greater intensity then the bands obtained from thigh muscles in both the varieties (Figure 1B) and (Table 1). Bands of μ-calpain from breast muscle were clearly visible only up to 6 h for both the varieties, but bands for thigh muscle were visible only at initial period, and were autolyzed at 6 h (Figure 1C & D). In the case of m-calpain the proteolytic activity was completely stable even upto 24 h (Figure 1a).

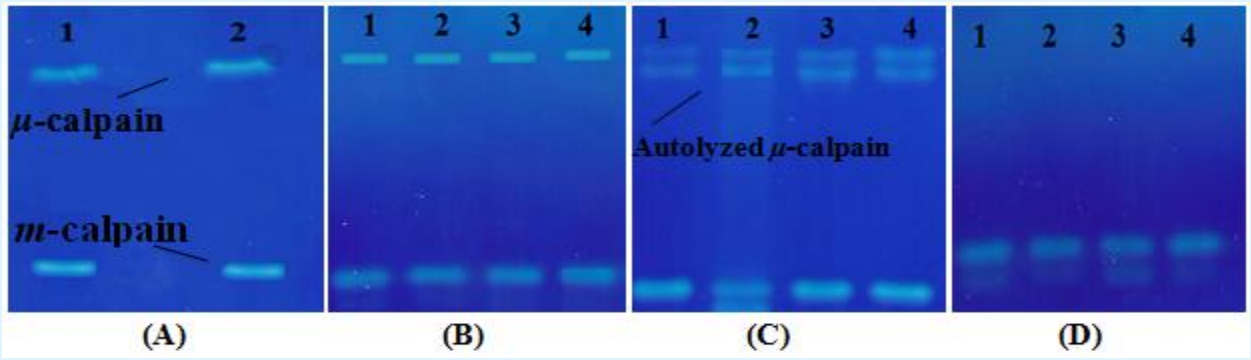

Figure 1: *μ*- and *m*-calpains on casein gel
(A) Lane-1: Swetambari Blood; Lane-2: Pearl Blood.
(B) 0 h: Lane-1: Swetambari Breast; Lane-2: Swetambari Thigh; Lane-3: Pearl Breast; Lane-4: Pearl Thigh.
(C) 6 h: Lane-1: Swetambari Breast; Lane-2: Swetambari Thigh; Lane-3: Pearl Breast; Lane-4: Pearl Thigh.
(D) 24 h: Lane-1: Swetambari Breast; Lane-2: Swetambari Thigh; Lane-3: Pearl Breast; Lane-4: Pearl Thigh.

Guneafowl	Muscle Type	Post-mortem aging time		
		0 h	6 h	24 h
μ-calpain				
Swetambari	Breast	0.47 ± 0.05^{cD}	0.21 ± 0.02^{bD}	0.08 ± 0.04^{aD}
	Thigh	0.29 ± 0.03^{cB}	0.13 ± 0.01^{bB}	0.06 ± 0.05^{aB}
Pearl	Breast	0.36 ± 0.04^{cC}	0.19 ± 0.03^{bC}	0.07 ± 0.01^{aC}
	Thigh	0.25 ± 0.02^{cA}	0.09 ± 0.04^{bA}	0.05 ± 0.03^{aA}
m-calpain				
Swetambari	Breast	2.01 ± 0.06^{aA}	1.96 ± 0.02^{aA}	1.93 ± 0.04^{aA}
	Thigh	1.98 ± 0.05^{aA}	1.88 ± 0.03^{aA}	1.85 ± 0.01^{aA}
Pearl	Breast	1.97 ± 0.02^{aA}	1.99 ± 0.06^{aA}	1.91 ± 0.05^{aA}
	Thigh	1.89 ± 0.04^{aA}	1.84 ± 0.03^{aA}	1.81 ± 0.02^{aA}
Calpastatin				
Swetambari	Breast	1.09 ± 0.01^{bD}	0.89 ± 0.04^{aD}	0.71 ± 0.03^{aD}
	Thigh	0.92 ± 0.02^{cB}	0.79 ± 0.03^{bB}	0.62 ± 0.01^{aB}
Pearl	Breast	0.99 ± 0.04^{cC}	0.72 ± 0.02^{bC}	0.53 ± 0.03^{aC}
	Thigh	0.81 ± 0.03^{cA}	0.69 ± 0.05^{bA}	0.47 ± 0.04^{aA}

Table 1: Changes in Calpains and Calpastatin Activity* During Post-Mortem Maturation at 4 ± 1 °C
n = 20; Mean ± S.E with different superscript row-wise (small letter) and column-wise (capital letter) differ significantly ($P < 0.05$).
*Unit: Units/g

Similar results were reported for chicken Pectoralis muscle at different time intervals [7] and complete absence of μ-calpain was found at 24 h post-mortem maturation. However, the studies conducted for lamb Longissimus dorsi muscle showed absence of μ-calpain at 72 h post-mortem [18], while m-calpain remained stable in both the studies. These studies hence conclude that μ-calpain present in guinea fowl species are less stable and shows rapid proteolytic activity, explaining the fact why avian muscles tenderize rapidly than there mammalian counterparts.

Identification of Calpains and Calpastatin in SDS-PAGE Analysis

SDS-PAGE analysis was mainly conducted to determine the presence of calpastatin in the crude extracts. Result depicted in Figure 2 indicates that the fractions eluted with 200 mM and 400 mM NaCl showed similar pattern of prominent bands for breast and thigh muscle samples from Swetambari and Pearl varieties.

There was a clear presence of prominent bands at molecular weight of 78, 76, 75 and 66 kDa for catalytic subunits and 30, 28 and 25 kDa for regulatory subunits in both muscle and blood samples indicating native and autolyzed forms of μ- and m-calpains. The 100 mM eluted fraction of blood samples showed completely different band patterns to that of 200 and 400 mM NaCl fractions. In both the varieties prominent bands were identified at molecular weight of 78, 66, 30, 28 and 25kDa for blood samples. However for breast and thigh muscle samples the bands were visualized at 100, 78, 66, 50, 40, 30, 28, and 25 kDa. The elution pattern obtained for 100 mM fraction showed contradictory result to the earlier findings in lamb and beef muscles [6, 18, 19]. This variation of band pattern could be due to the fact that calpastatin contains 4 repeating, marginally homogenous domains I, II, III and IV, and in addition to N-terminal domain, the L-domain that varies in size due to alternative splicing events and different strategies of transcription, although it (L-domain) has no inhibitory activity.

During maturation process (Figure 2), similar band pattern as mentioned above was observed but intensity of band clearance decreased with increase of maturation time and there was minor presence of bands at 24 h post-mortem. These results were in lieu with the spectrophotometric activity analysis which showed negligible activity of calpains and calpastatin after 24 h. Similar, results were also reported by Lee, et al. [7] in chicken breast muscle, and according to them,

predominant band at molecular weight 110 kDa disappeared in between 3 and 6 h of post-mortem holding whereas the band of 75 kDa decreased after 6 h. Moreover they also stated the presence of 30 kDa band increased after 6 h, indicating the hydrolysis of troponin T by calpain, which was in lieu with our study. Similar, findings were also reported for goat leg muscles [11].

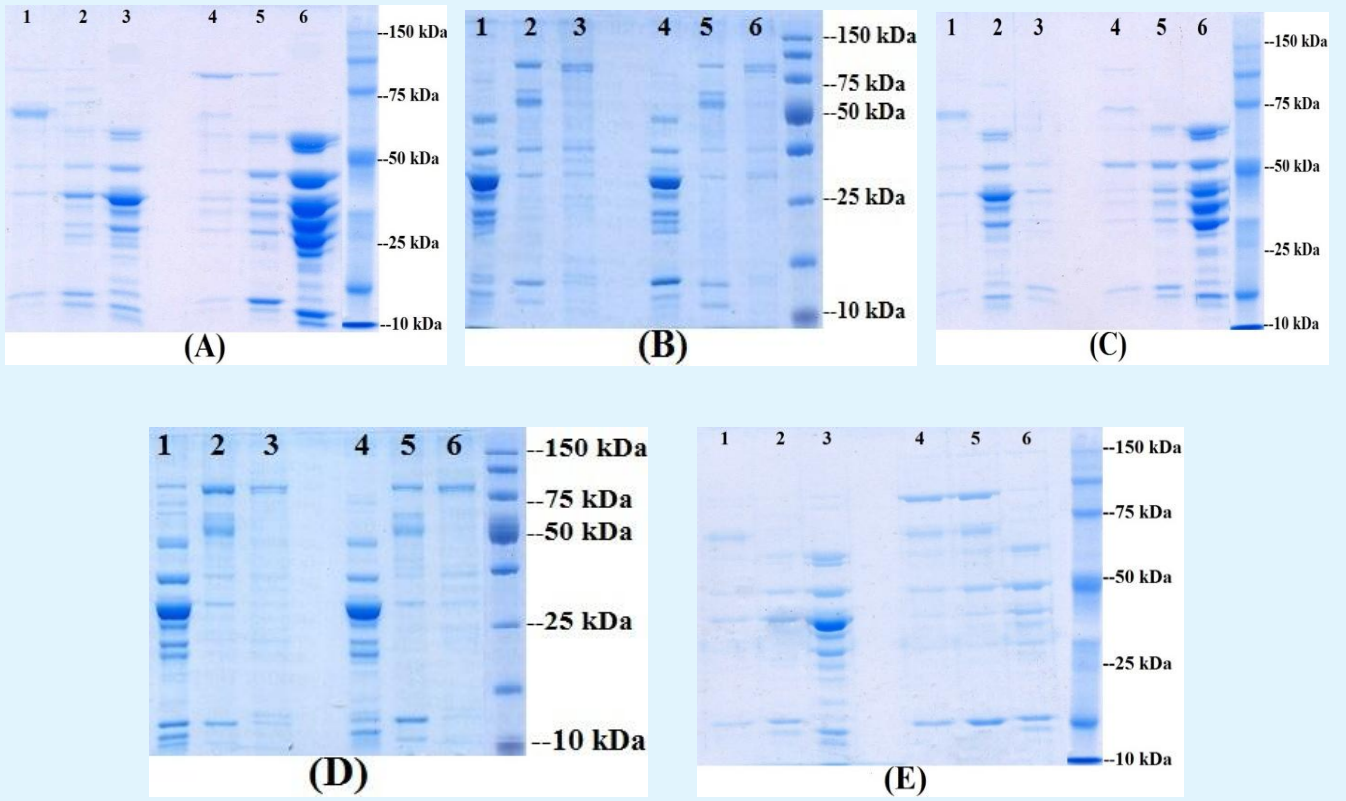

Figure 2: Calpains and calpastatin on SDS-PAGE gel.
(A) Lane-1: Swetambari blood μ-calpain; Lane-2: Swetambari blood *m*-calpain; Lane-3: Swetambari blood calpastatin; Lane-4: Pearl blood μ-calpain; Lane-5: Pearl blood *m*-calpain; Lane-6: Pearl blood calpastatin.
(B) 0 h Swetambari: Lane-1: Breast calpastatin; Lane-2: Breast *m*-calpain; Lane-3: Breast μ-calpain; Lane-4: Thigh calpastatin; Lane-5: Thigh *m*-calpain; Lane-3: Thigh μ-calpain.
(C) 24 h Swetambari: Lane-1: Breast μ-calpain; Lane-2: Breast calpastatin; Lane-3: Breast *m*-calpain; Lane-4: Thigh μ-calpain; Lane-5: Thigh *m*-calpain; Lane-3: Thigh calpastatin.
(D) 0 h Pearl: Lane-1: Breast calpastatin; Lane-2: Breast *m*-calpain; Lane-3: Breast μ-calpain; Lane-4: Thigh calpastatin; Lane-5: Thigh *m*-calpain; Lane-3: Thigh μ-calpain.
(E) 24 h Pearl: Lane-1: Thigh μ-calpain; Lane-2: Thigh *m*-calpain; Lane-3: Thigh calpastatin; Lane-4: Breast μ-calpain; Lane-5: Breast *m*-calpain; Lane-3: Breast calpastatin.

Calpains and Calpastatin Activity in Blood and Post-Mortem Muscle

Casein zymography results clearly indicated that μ-calpain retained its activity upto 6 h for breast muscle samples and after that it was completely autolyzed, these variations were detected through spectrophotometric activity analysis. It was found that the activity of μ-calpain, m-calpain and calpastatin from blood samples of Swetambari was significantly ($P<0.05$) higher than μ-

calpain, m-calpain and calpastatin of Pearl. But, it was found that activity of these enzymes was higher for muscle samples in both the varieties. Again the activity of calpains and calpastatin was higher for Swetambari than that of Pearl varieties. However, post-mortem activity analysis of μ-calpain (Table 1) from breast and thigh muscle decreased by 93 and 84 % for Swetambari whereas it decreased by 88 and 80% for Pearl at 24 h. Post-mortem maturation has very little effect on activity analysis of m-calpain (Table 1) from breast and thigh muscle as it decreased by 4 and 7% for Swetambari whereas by 3 and 6% for Pearl at 24 h. Calpastatin activity of breast and thigh muscles also varied slightly during post-mortem maturation and decreased by 13 and 16% for Swetambari and 11 and 16% for Pearl at 24 h. Activity analysis results were in comparison with the studies conducted for lamb Longissimus muscle [18], and according to them, proteolytic activity of μ-calpain remained almost unaffected for more than 72 h.

Changes in pH and W-B shear force value (WBSFV)

It has been observed that the pH value of breast and thigh muscle samples decreased significantly (P<0.05) with the increase of post-mortem maturation. However, just after slaughter it was reported that the pH of breast muscle was higher than that of thigh muscle samples for both the varieties. (Table 2) depicts significant (P<0.05) decrease in pH value of breast and thigh muscle up to 6 h for both the varieties. This significant decrease in pH is due to the breakdown of glycogen to lactic acid. The pH value reached at 24 h post-mortem is termed as ultimate pH after which pH did not change significantly and the muscle reaches rigor [7]. Maddock, et al. [20] have also reported that with the increase in post-mortem maturation the pH value decreased, and due to this, the calpains started to precipitate resulting in their decreased activity.

Guneafowl	Muscle type	Post-mortem aging time		
		0 hr	6 hr	24 hr
pH value				
Swetambari	Breast	6.59 ± 0.03^{bD}	6.09 ± 0.07^{aD}	5.98 ± 0.01^{aA}
	Thigh	6.46 ± 0.04^{bC}	5.99 ± 0.02^{aC}	5.95 ± 0.03^{aA}
Pearl	Breast	6.31 ± 0.03^{bB}	5.81 ± 0.07^{aB}	5.89 ± 0.05^{aA}
	Thigh	6.17 ± 0.05^{bA}	5.68 ± 0.03^{aA}	5.75 ± 0.04^{aA}
WBSFV (Kg/cm^2)				
Swetambari	Breast	4.62 ± 0.05^{bD}	3.96 ± 0.04^{aD}	3.59 ± 0.05^{aA}
	Thigh	3.81 ± 0.02^{bB}	2.98 ± 0.05^{aB}	2.57 ± 0.03^{aA}
Pearl	Breast	4.23 ± 0.07^{bC}	3.68 ± 0.04^{aC}	3.48 ± 0.06^{aA}
	Thigh	3.56 ± 0.03^{bA}	2.65 ± 0.07^{aA}	2.51 ± 0.02^{aA}

Table 2: Changes in pH and Warner-Bratzler shear force value of during post-mortem maturation at 4±1 °C.
n = 20; Mean ± S.E with different superscript row-wise (small letter) and column-wise (capital letter) differ significantly (P < 0.05).

Warner-Bratzler shear force value was measured to determine the role of μ-calpain mediated post-mortem tenderization on the texture quality of meat. WBSF value for breast and thigh muscles decreased significantly (P<0.05) during the post-mortem maturation, with breast muscle showing higher value than thigh muscle (Table 2). It has been deduced that Swetambari has higher value of WBSF than Pearl. These variations have significant effect on tenderness of meat. WBSF values were in relation with zymography and activity analysis results, showing the optimum tenderization at 6 h post-mortem. Moreover these findings were supported by the analysis conducted by Liu, et al. [21] who also stated that meat kept for post-mortem maturation showed a significant (P < 0.05) decrease in shear force value with increase in maturation time.

Conclusions

Proteolytic activity of different enzymes of calpain system in blood and muscle samples was easily identified using casein zymography and SDS-PAGE analysis in two different guinea fowl varieties. Purification and quantification of calpains and calpastatin was performed with high selectivity using Biospectrophotometer. This study paves the way to predict quality of meat by analyzing blood samples just before the day of slaughter. The μ-calpain mediated post-mortem maturation was

optimized at 6 h and was found to be on the higher side in Swetambari than in Pearl varieties of guinea fowl birds.

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