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Influence of Calpain Mediated Post-Mortem Ageing On Quality of Broiler Breeder Breast Fillets during Holding at Room Temperature

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ABSTRACT

The aim of this study was to determine the activity of different calpains (μ and m) and calpastatin in broiler breeder breast fillets to understand their influence on ageing during holding at (27±2) °C. Both of the enzymes were extracted and they were subjected to casein zymography analysis. The purified enzymes were separated using anion exchange column chromatography, and their presence was confirmed by SDS-PAGE analysis. Casein Zymography results revealed the presence of μ-and m-calpains activity in breast fillets sample extracts. Though it has been observed that the band intensity keep on decreasing with the increasing ageing time which indicate the decrease in activity of these enzymes. The pH and Warner-Brazler Shear Force (WBSF) values were also decreased with the increase in ageing time while Lovibond tintometer colour, TBARS values, FFA contents and peroxide values were not much affected. Thus, it was concluded that both these enzymes are present in the muscle sample but that were autolyzed with the increase of aging. On the basis of results obtained it might be concluded that the optimum ageing time for broiler breeder breast fillets was optimized 4h at room temperature holding at(27±2) °C.

Key words: *Calpains, Calpastatin, Casein zymography, Breast fillets*

INTRODUCTION

Toughness of broiler breeders is the major limiting factor affecting consumer's acceptability in the market, the problem of consumer dissatisfaction will be solved only by solving the problem of unacceptable variation in meat toughness. It has been reported that as the animal matures, fiber hypertrophy is accompanied by maturation of the endomysium, perimysial thickness and the formation of non reducible cross-links between the collagen molecules. The inferior quality, such as toughness in meat, is primarily due to increased cross-linking in the connective tissue of older animals. Many attempts have been made to tenderize meat from unproductive animals using mechanical or artificial tenderizing techniques[23], but limited studies were carried out to explore advantages of natural tenderization process which is accompanied by endogenous enzymes during post-slaughter period.

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The tenderness of meat can be improved by efficient handling and processing on post-mortem holding before use. But several studies revealed that the meat tenderization process is complex mechanism which could be affected by several pathways including pre- and post-slaughter factors, and within these factors, it is likely that ultimate tenderness is mainly determined by the extent of proteolysis of key target cytoskeleton proteins of muscle fibres and the alteration of muscle structure[13].

Calpains (*μ* & *m*), calcium-dependent proteases, have been recognized as key players in post-mortem tenderization of skeletal muscle. Calpastatin, another endogenous enzyme of calpain system is inhibitory to calpains and is widely distributed in muscle tissues. However, there is very little information on the effect of calpain mediated post-mortem ageing on the quality of pre-cooked breast fillets from breeder broilers. So, efforts were made to explore the effect of calpain mediated post-mortem ageing on the quality of breast fillets from broiler breeders by applying post-mortem aging method.

MATERIALS AND METHODS

Chemicals and reagents

Purified casein powder from bovine milk, dialysis tubing (12 kDa MWCO), DEAE-Sephacel anion exchanger and protease inhibitors (leupeptin hemisulphate, ovomucoid, phenyl-methane sulphonyl fluoride) were procured from Sigma-Aldrich, St. Louis, USA. Precision plus protein All Blue Standards (10-250 kDa) and Econo-Column were obtained from Bio-Rad Laboratories, Lucknow.

Sample collection

A total –breast muscle samples (Broiler Breeder, above 50 wks of age) were collected from Experimental Poultry Processing Plant of ICAR-Central Avian Research Institute, Izatnagar, Bareilly.

Purification and separation of μ- and m-calpains and calpastatin from sample extract

Processing and extraction of samples for extraction of μ–and m–calpains and calpastatin was done as per the method developed by Biswas *et al*[4].

Casein Zymography analysis

Methodology as reported by Biswas *et al*[4]., for the detection of calpains was followed. Casein Zymography method is based on the principle that casein molecules present in the zymography gel are catalyzed by the calpains in the presence of Ca^{++} ion in the solution. The casein gels (thickness 0.75 mm, 4.2 ml gel) were prepared in the gel cassette assembly (Mini–Protean® Tetra System, Bio-Rad) as per the protocol provided with the equipment. The separating gels (10 % with 0.21 % casein) and stacking gel (5 % without casein) were prepared as per standard protocol.

SDS-PAGE analysis

SDS–PAGE analysis was carried out as per the protocol described by Laemmli[20] (1970) with slight modifications. The gels were cast in Mini–Protean® Tetra Gel System (Bio–Rad, India).

Determination of calpains and calpastatin activity

Enzymatic activities of calpains (*μ* and *m*) and calpastatin were determined using casein as a substrate as described by Dayton *et al*[9]., with slight modification. Total activity was calculated by multiplying Ca^{++} dependent proteolytic activity by the dilution factor.

CDP Activity (Units/g) = (A_{278} $CaCl_2$ buffer – A_{278} EDTA buffer) x 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.

Determination of pH, Water holding capacity (WHC) and W-B shear force value (WBSFV)

The pH of breast fillets (n=6) was determined[29] a Bench top digital pH meter equipped with a glass electrode and automatic temperature sensors. Water holding capacity was determined by press method[31].

Water holding capacity (%) = (Weight of meat sample–Actual weight of meat sample) x 100 / Weight of meat sample

The shear force value of the breast and thigh meat samples was measured following the method of Berry *et al*[2].

Lovibond Tintometer colour

The colour profiles of raw and precooked breast fillets were measured using Lovibond tintometer (Model F, Greenwich, U.K.).

Thiobarbituric acid reacting substances (TBARS) numbers

The extraction method described by Witte *et al*[32]., was used for determination of TBARS value.

Free fatty acids (FFAs)

The method as described by Koniecko[18] was followed for determination of free fatty acid (FFA) contents. The quantity of potassium hydroxide required for titration was recorded and calculated as follows:

$$\text{Free fatty acid (FFA)} = \frac{(0.1 \times \text{ml } 0.1\text{N alcoholic KOH} \times 0.282)}{\text{Wt. of sample (g)}} \times 100$$

Peroxide value

The peroxide value was measured as per procedure described by Koniecko[18]. The peroxide value (meq/kg of the meat) was calculated as per the following formula:

$$\text{PV (meq/kg sample)} = \frac{(0.1 \times \text{ml } 0.1\text{N sodium thiosulphate})}{\text{Wt. of sample (g)}} \times 1000$$

Myofibrillar Fragmentation Index (MFI)

The myofibrillar fragmentation index (MFI) was determined in broiler breast fillet samples as described by Davis *et al*[8].

Protein extractability

Salt extractable proteins (SEP) and water extractable proteins (WEP) were estimated as per the method of Kang and Rice[15].

Microbiological analysis

Standard plate count, Total coliforms count, Psychrotrophic count, *E. coli* count, *Staphylococcus* spp., *Salmonella* spp. and yeast and mould count (Y&M) in the samples were enumerated as per the method described by American Public Health Association[1].

Statistical Analysis

Experimental data were analysed statistically using standard software package as mentioned by Snedecor and Chochran[28]. Means of calpains (μ- and *m*) and calpastatin activity and other data relating to physic-chemical analysis were evaluated using one-way ANOVA.

RESULTS AND DISCUSSION

Casein zymography

Casein acts as a very good *in-vitro* substrate for proteolytic activity of calpain, so it was incorporated in zymography study (Figure 1). Results showed that extracts prepared from breast muscle samples had ample proteolytic activity since clear bands of μ- and *m*-calpain were visualized on zymogram. However a decreasing trend for proteolytic activity with the increase in post-mortem ageing was observed. The bands produced by μ-calpain were visible only up to 4 h and were completely absent at 6 h indicating substantial degradation of this enzyme. In case of *m*-calpain, it was clearly visualized at 36 h indicating good stability of this enzyme in post-mortem breast muscle. The results obtained are in accordance with the earlier findings of Biswas *et al*[5]., in breast muscle from White Leghorn. Similar findings were also reported by Huang *et al*[14]., in chicken breast muscle.

Identification of calpains and calpastatin in SDS-PAGE analysis

Results illustrated in Fig. 2 & 3, the fractions eluted with 200 mM and 400 mM NaCl showed similar band pattern for broiler breast muscle. The presence of prominent bands at molecular weight of 78 kDa and 76 kDa for catalytic subunits and 28 kDa and 25 kDa for regulatory subunits in muscle samples indicate native and autolyzed forms of μ- and *m*-calpains, respectively. The 100 mM eluted fraction showed completely different band

pattern as the prominent bands were observed at 66 kDa and 33 kDa.

Calpain and calpastatin activity in post-mortem breast muscle

Results presented in Fig. 1 indicate that μ-calpain remained in its native form up to 4 h in post-mortem muscle sample and it was completely absent at 6 h. Table 1 showed that activity of μ-calpain was considerably higher just after slaughter and decreased to about 85 % at 6 h post-mortem. Similarly calpastatin activity decreased by 62 % at 6 h post-mortem, however *m*-calpain remained stable since its activity decreased by mere 16 % which was clearly visualised through casein zymography analysis. In general, activity found in this study varied little from earlier findings reported by Biswas *et al*[4]., for turkey breast muscle stored at refrigeration temperature, however, the study conducted by Northcutt *et al*[24]., showed lower value of calpains for turkey.

SDS–PAGE analysis

Results showed that the appearance of the bands for μ--calpain and calpastatin decreased with the increase in time of post-mortem aging (Fig. 3). Assay analysis for calpastatin from breast muscle showed a decrease in activity by 30% at 4 h ageing at room temperature (Table 1). These changes in the activity of calpains and calpastatin may be due to decline of muscle pH during post-mortem aging. Similar findings were also reported by Veiseth *et al*[30]., in beef and lamb muscles.

Changes in pH, Water holding capacity (WHC) and W-B shear force value (WBSFV)

Results presented in Table 1 observed that μ-calpain dependant postmortem aging of broiler breeder breast muscle was completed at 4 h and during that time pH of the sample was recorded to be 5.75, and thereafter, it decreased gradually. Various studies have revealed that breast muscle from chicken species developed post-mortem aging as early as 2h or even after 8 h. This indicated that the post-mortem aging time relating to development of pH is poorly defined[12]. At slaughter, the mean pH value of the breast muscles was 6.00, and it gradually reduced up to 8 hrs. Therefore, post-mortem aging showed significant ($P<0.05$) influence on changes in muscle pH. The rate of pH decline in breast muscle was most rapid over the first 4 h of the post-slaughter period, and significant differences ($P\leq0.05$) was found among times required for aging. The results obtained in this study are consistent with the earlier findings as the breast muscle of chicken exhibits accelerated rigor mortis compared with beef or lamb muscles[25].

WHC of breast fillets were decreased ($P\leq0.05$) with the increase of ageing time, the value ranged from 24.72 % at 30 min post-slaughter to 12.67 % at 8 h during holding at (27±2) ºC. The WHC however decreased significantly ($P\leq0.05$) only after 2 h of ageing at room temperature. Decreasing of WHC with the increase of ageing time could be attributed to the decrease of pH which leads to denaturation and precipitation of some of the sarcoplasmic proteins on actin and myosin filaments. Similar to this study changes of WHC due to post-mortem ageing were reported by Mendiratta *et al*[23].

The results of WBSFV of post-mortem breast fillets are shown in Table 1. It has been observed that immediately after slaughtering of birds the WBSFV was maximum in breast fillets muscle, whereas with the increase in aging time the WBSFV declined, confirming the increase in tenderness of muscle samples. These findings are coincided with the results of calpain activity in post-mortem muscle (Table 1). Similar findings were also reported by Liu *et al*[22]., and according to them holding of muscle for postmortem aging showed a significant decrease in WBSFV with increase in storage time.

Lovibond Tintometer colour

The colour coordinates of *a*- (redness) and *b*- (yellowness) values indicated that they were decreased and increased respectively with the increase of ageing times. Redness value decreased from 0.16 to 0.08 from 30 min to 8 h of post-mortem ageing at (27±2) ºC. During periods of ageing decease in *a*-value seems to be due to formation of metmyoglobin which

leads to dark discolouration of breast fillets. The findings of this study are in general agreement with the reports of Le Bihan-Duval *et al*[21]. Yellowness (*b*-value) showed non-significant increasing trend from 0 to 4 h of ageing but from 4 to 8 h of ageing, b-value showed significant increasing trends (Table 1).

Thiobarbituric acid reacting substances (TBARS) value

The extent of lipid oxidation of breast fillets, as measured by MDA (Malondialdehyde) formation, was increased with the increase of post-mortem ageing periods but the values were differed non-significantly (P≥0.05). MDA contents were increased from 0.28 mg MDA/kg at 30 min to 0.32 mg MDA/kg at 6 h during ageing of breast fillets shows that oxidative deterioration started from the initial period (30 min) and was increased until 8 of ageing (Table 1). The increase of MDA contents of breast fillets from spent broiler chicken with the increase of ageing time could be due to temperature induced lipid oxidation, which is attributed to disruption of muscle cell membranes that facilitate the interaction of unsaturated fatty acids with pro-oxidant substances[6]. However, the results in this study TBASRS values were far below the threshold level of 2 mg MDA/kg of meat.

Free fatty acid

Free fatty acid (FFA) content in meat determines the fat status and quality of the muscle foods and expressed as percent of oleic acid. Within few hours of onset of ageing process formation of free fatty acids convene but their values were not significant (P≥0.05) (Table 1). However, gradual increasing trends of FFA contents were observed with the increase of ageing time could be attributed to the release of more FFAs from fillets of the fatty broilers, and on subsequent ageing due to enzymatic or microbial lipolysis of fat. Similar trends were found by Kripriyalini[19] in turkey breast meat and by Biswas *et al*[3]., in chicken meat products. FFA determination alone did not provide guide for acceptability of the meats, but support as suitable quality indicator for oxidative changes of fat.

Peroxide value (PV)

Lipid oxidation is the principal cause of deterioration of the quality of poultry meat and also important determination of shelf life of meat and meat products. Post-mortem ageing at (27±2) °C showed non-significant (P≥0.05) effect on PV of breast fillets. As expected PV was lowest at 30 min and there was very little increase of PV during post-mortem ageing at room temperature. At the end of ageing, the PV value was found around 0.15 meq/kg (Table 1). This result agreed with findings of Ramzija *et al*[27]., who reported that peroxide values of 7.77 meq/kg of fat in chicken breast muscle, but these authors did not reported PV in meat on fresh basis. However, the increase of PVs with the increase of ageing time could be attributed to presence of residual oxygen in the packages[17].

Myofibrillar fragmentation Index (MFI)

Myofibrillar fragmentation index (MFI) is a useful indicator of post-mortem proteolysis of muscle fibers, was increased significantly (P≤0.05) with the increase of post-mortem ageing times during holding at (27±2) ºC temperatures. The MFI was increased with ageing at room temperature (27±2) ºC until 8 h. Similar observations were also reported by Obanor *et.al*[25]. Kim *et.al*[16]., reported that pre-rigor chicken breast muscle salted with 2% NaCl showed lower MFI value than post-rigor chicken breast muscle salted with 2% NaCl.

Protein extractability (Water and salt soluble proteins)

Post-mortem ageing at room temperature, WSP and SSP were increased and decreased (P≤0.05) respectively up to 24 h (Table 1). During initial stage of ageing, the higher protein extractability for SSP could be due to myosin and actin molecules of muscle fibres were in free form and when they progress toward rigor state the availability of free myosin and actin molecules were minimum thereby showed lower SSP at 24 h of ageing. The increased of WSP with the increase of ageing periods could be correlated with to denaturation of sarcoplasmic proteins at early post-mortem due to rapid post-mortem glycolysis as a result of decreased in muscle

pH, though proteolysis of some key cytoskeletal proteins of muscle cells were observed. Similar findings were reported by Eady *et al*[10]., but a decrease in sarcoplasmic protein solubility with aging has been observed in beef[7]. So, it seems that post-mortem aging of chicken breast fillets had direct influence on protein extractability.

Microbiological study

Result of microbiological evaluation is presented in Table 2. It has been observed that the values of Standard plate count, Total coliform count, Psychrotrophic plate count, *E. coli* count and yeast and mould count (log_{10} cfu/g) increased significantly (P≤0.05) during storage between T_1 and T_2. Standard plate count was 3.08 and 4.05 log_{10} cfu/g at 0 and 4 hr ageing at (27±2) °C ageing. Total coliform count ranged from 0.54 and 0.88 at 0 hr and 4 hr ageing (27±2) °C. Data for *E. coli* count were also significantly different between T_1 and T_2. Psychotrophic plate count increased significantly during the storage period but still these values were comfortably in the safety levels set by FSSAI. *Staphylococcus aureus* spp. count and *Salmonella spp*. was not detected in any of the samples. Yeast and Mould counts were 0.25 and 0.85 log_{10} cfu/g at 0 hr and 4 hr ageing at room temperature. These organisms are known as indicators and their presence is an indication that the meat samples were exposed to conditions that might introduce pathogenic organisms[26]. The increase of SPC with the increase of ageing time in breast muscle samples might be attributed to the availability of more readily utilizable nutrients in muscle by the bacteria. Elmali and Yaman[11] reported high level of *Salmonella*, *Staphylococcus*, coliform, aerobic mesophiles in raw turkey meat samples. Significant (P≤0.05) variations of microbial counts amongst breast fillets could be attributed to variations in slaughtering operations, contamination during evisceration of the internal organs, abattoir hygiene, conditions of the storage, personal hygiene. It was observed that microbial counts in breast fillets were increased with the increase of ageing time but they are far below the acceptable as recommended by FSSAI.

Table 1: Effect of calpain mediated post-mortem ageing on quality of breast fillets during holding at room temperature (27 ± 2 °C)

Parameters#	Post-mortem ageing times (Hrs)				
	0.5	**2**	**4**	**6**	**8**
μ-calpain (Units/g)	0.74 ± 0.003^e	0.55 ± 0.007^d	0.35 ± 0.003^c	0.11 ± 0.006^b	0.03 ± 0.006^a
m-calpain (Units/g)	2.84 ± 0.009^e	2.78 ± 0.009^d	2.59 ± 0.006^c	2.38 ± 0.009^b	2.17 ± 0.012^a
Calpastatin (Units/g)	1.77 ± 0.007^e	1.63 ± 0.007^d	1.23 ± 0.006^c	0.66 ± 0.006^b	0.10 ± 0.006^a
pH	6.00 ± 0.10^b	5.97 ± 0.08^b	5.75 ± 0.06^a	5.70 ± 0.06^a	$5. 63 \pm 0.03^a$
WHC (%)	24.72 ± 1.66^b	22.67 ± 0.61^b	16.67 ± 1.45^a	14.05 ± 1.46^a	12.67 ± 1.09^a
WBSFV (Kg/cm²)	2.85 ± 0.16^d	1.73 ± 0.15^c	1.23 ± 0.09^b	1.07 ± 0.08^{ab}	0.80 ± 0.06^a
Redness (*a*-value)	1.16 ± 0.02^b	1.13 ± 0.02^a	1.10 ± 0.02^a	1.13 ± 0.03^a	1.08 ± 0.04^a
Yellowness (*b*-value)	1.07 ± 0.02^a	1.09 ± 0.03^a	1.11 ± 0.02^{ab}	1.13 ± 0.13^b	1.15 ± 0.02^b
TBARS (mg MDA/Kg)	0.28 ± 0.111	0.29 ±0.009	0.30 ±0.020	0.32 ± 0.004	0.34 ±0.031
FFA contents (%)	0.04 ± 0.005^a	0.04 ± 0.003^a	0.03 ± 0.003^a	0.08 ± 0.003^b	0.08 ± 0.002^b
Peroxide value (meq/kg)	0.15 ± 0.02	0.15 ± 0.01	0.15 ± 0.02	0.16 ± 0.01	0.16 ± 0.02
MFI (%)	10.70 ± 0.36^a	18.48 ± 0.47^b	28.76 ± 1.29^c	29.53 ± 2.07^c	31.93 ± 1.46^c
WSP (%)	12.12 ± 0.31^a	14.12 ± 0.47^b	17.93 ± 1.47^c	18.87 ± 2.44^c	19.59 ± 2.46^c
SSP (%)	25.03 ± 1.51^b	23.42 ± 0.80^{ab}	22.28 ± 0.36^a	22.18 ± 0.54^a	21.35 ± 0.49^a

n = 6; Mean ± S.E. with different superscript row-wise (small letter) differ significantly (P<0.05).

#WHC = Water holding capacity, WBSFV = Warner-Bratzler shear force value, TBARS = 2- thiobarbituric acid reacting substances, FFA = Free fatty acid, MFI = Myofibrillar fragmentation index , WSP = Water soluble protein, SSP = Salt soluble protein

Table 2: Microbiological* quality of breast fillets at optimum ageing time during holding at room temperature (27 ± 2 ºC)

Parameters	Post–mortem (PM) ageing time		
	T_1	T_2	T_3
Standard plate count	3.08 ± 0.06^{a}	4.05 ± 0.17^{b}	3.00 ± 0.39^{a}
Total Coliform count	0.54 ± 0.03^{a}	0.88 ± 0.07^{b}	0.76 ± 0.10^{a}
Psychrotrophic plate count	1.48 ± 0.21^{a}	2.15 ± 0.36^{b}	1.78 ± 0.20^{a}
E. coli count	0.25 ± 0.05^{a}	0.6 ± 0.11^{b}	0.55 ± 0.15^{a}
Staphylococcus aureus spp. count	ND	ND	ND
Salmonella count	ND	ND	ND
Yeast & mould count	0.25 ± 0.25^{a}	0.85 ± 0.29^{b}	0.45 ± 0.25^{a}

*Unit: $\log_{10}$ cfu/g of sample, n = 4, ND = Not detected, Mean ± S.E. with different superscript row-wise small letter differ significantly (P < 0.05).

T_1 = Without ageing, T_2 = After 4 h of ageing at room temperature (27 ± 2 ºC)

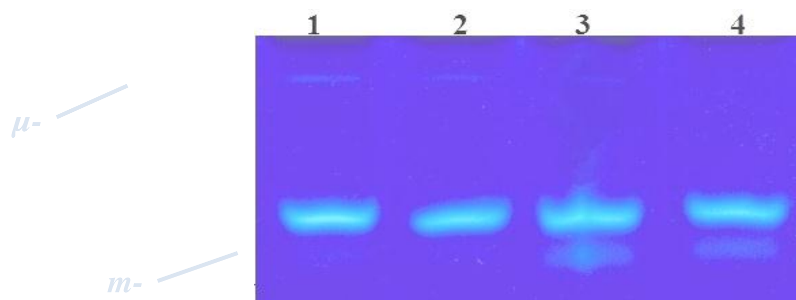

Plate 1: Casein zymogram shows clear bands of *μ*- and *m*-calpains of breast muscle from broiler breeder during ageing at room temperature (27 ± 2 ºC), Lane-1: **0 h** breast muscle, Lane-2: **2 h** breast muscle, Lane-3: **4 h** breast muscle, Lane-4: **6 h** breast muscle.

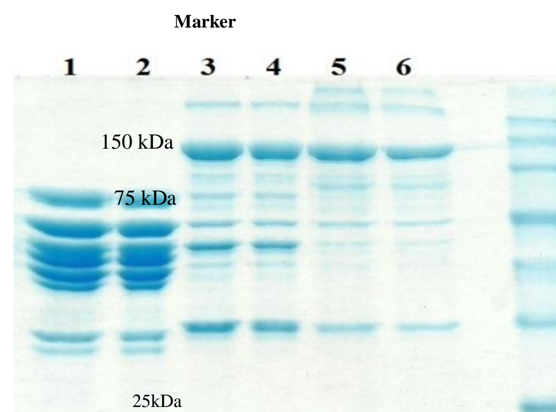

Plate 2: SDS-PAGE shows clear bands of calpains and calpastatin of breast muscle from broiler breeder immediately after slaughter (0 h), Lane-1 & 2: Calpastatin, Lane-3 & 4: *m*-calpain, Lane-5 & 6: *μ*-calpain, [Protein Markers: Novagen Perfect Protein Markers (10 – 150 kDa)

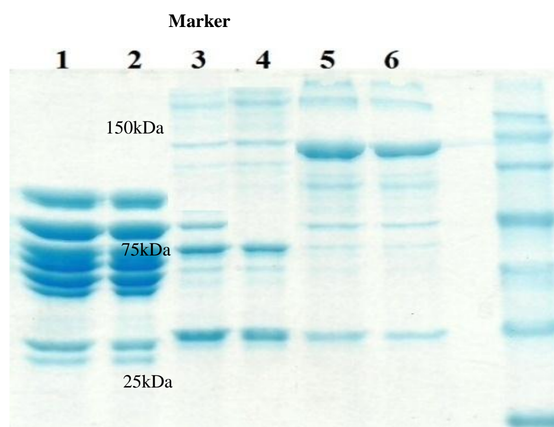

Plate 3: SDS-PAGE shows clear bands of calpains and calpastatin of breast muscle from broiler breeder after 6 h of ageing at room temperature (27 ± 2 ºC), Lane-1 & 2: Calpastatin, Lane-3 & 4: *m*-calpain, Lane-5 & 6: *μ*-calpain, [Protein Markers: Novagen Perfect Protein Markers (10 – 150 kDa)]

CONCLUSION

Casein Zymography is a simple method successfully utilized for accurate determination of calpains and calpastatin in spent broiler breast fillets samples. In addition, simultaneous analysis of *μ*- and *m*-calpains as well as calpastatin was also determined through biochemical assay to understand their association in proteolytic activity in the presence of calpastatin, a potential inhibitor of calpains. Besides these Warner-Bratzler shear force value (WBSFV), myofibrillar fragmentation index (MFI) and protein extractability of the fillet samples were determined. Additional parameters like changes in pH, Lovibond tintometer colour, free fatty acid contents, peroxide value, TBARS value and microbial quality were evaluated at different time intervals as mentioned earlier. The post-mortem aging time for chicken breast fillets were optimized at 4 h during holding at (27±2) ^{o}C.

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