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Influence of Calpain Mediated Post-Mortem Ageing on Quality of Broiler Breeder Breast Fillets during Refrigerated Holding at (4 ± 1) °C

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A B S T R A C T

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 (4±1) °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 24h at refrigerated (±1) °C holding.

Introduction

Amongst all poultry species, chicken meat, and in particularly, broiler meat is getting wider popularity in the global food market because of white meat, and has some desirable properties over red meat that is excellent flavour, high in protein but low fat content etc. It is also efficient converter of feed into animal protein of high biological value as compared to other livestock species. But selling of meat from broiler breeders is getting more challenging task by the farmers, because it produces tough meat which is less palatable than usual market weight broilers. As scope of income generation and profit margin is limited with the broiler breeders, so it is logical and meaningful to develop further processed, value added products to earn reasonable return. Processing of broiler breeders into further processed products could also help in sustaining the demand for broiler meat besides improving the market accessibility by the consumers.

Since toughness of broiler breeders is the major limiting factor affecting consumer's acceptability in the markets, the problem of

consumer dissatisfaction will be solved only by improving the tenderness of tough meat. The tenderness of meat can be improved by using mechanical tenderization techniques, but very less work has been carried out to explore the advantages of natural tenderization techniques, based on the action of key proteolytic enzymes like calpains and calpastatin. The calpain system comprises of calpains (μ and m) which is calcium dependent endogenous proteolytic enzymes, recognized as key player in post-mortem tenderization of meat. Calpastatin another proteolytic enzyme of calpain system acts as an inhibitor to calpains. Both calpain and calpastatin are widely distributed throughout the muscle cells. Considerable evidences suggest that calpains are the proteolytic enzymes that are responsible for post-mortem proteolysis and tenderness of meat, but it is still not clear whether both μ-and m-calpain are accountable for the process or μ-calpain alone is responsible. Many studies illustrated that μ-calpain and calpastatin gradually lose their activity during post-mortem ageing, but m-calpain is very stable and is partially autolyzed. Therefore, identification of these enzymes in tissue samples is often an essential component of different studies which aimed at explaining variation in tenderness of meat.

Some workers studied the identification, quantification and purification of calpains and calpastatin in post-mortem muscles of different species (Biswas, Tandon, and Sharma, 2016; Geesink and Koohmaraie, 1999a; Huang, Huang, Ma, Xu, and Zhou, 2012). But the determination of the influence of μ-calpain in the proteolysis of broiler breast meat during post-mortem aging, has least been explored.

Therefore in this study attempts have been made to identify calpains and calpastatin and their activity in post-mortem tenderization of breast muscle during holding at (4±1) ºC.

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. All other chemicals and reagents used in 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.

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. The birds were slaughtered as per standard slaughtering practices. Immediately after exsanguinations, the skin covering was removed and the breast muscles were excised. The samples were collected in self-sealing LDPE bags and transferred to laboratory under chilled (0-1 ºC) condition for application.

Processing and extraction of samples

The procedure for extraction of μ–and m–calpains and calpastatin was followed as per the method developed by Biswas, Kripriyalini, Tandon, Sharma, and Majumdar (2016). Freshly collected tissue (breast muscle) samples were cut in to fine pieces after being trimmed–off excessive connective tissues, fat and fascia. About 0.5 gm of finely cut muscle samples were homogenized with 6 volumes of

ice–cooled extraction buffer containing 50 mM tris–base (pH 8.3) 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 ovomucoid, 6 mg/l leupeptin] were added in the extraction buffer just before preparation of meat homogenate. The extracts were then centrifuged at 12000 rpm (15000g) for 20 min at 4 °C (Eppendorf 5427R, Germany). Supernatants were collected in a separate centrifuge tube and sediments were disposed–off. The collected supernatant was centrifuged once again as mentioned earlier and was later filtered and collected in a separate tube.

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

The purification and separation is important for identifying calpastatin, which is an important enzyme of calpain system that inhibits the activity of calpains. These were performed by dialysis of extract followed by column chromatographic techniques. 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. The supernatant was collected and filtered through a Whattman Filter paper No.1 and 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. DEAE–Sephacel column was equilibrated with equilibrium buffer (pH 7.4) comprising of 40 mM tris–base, 0.5 mM EDTA and 0.05 % (v/v) MCE. After three times washing with equilibrium buffer (3x20 ml), sample extract corresponding to 0.5 g of meat was loaded on a column having 5 cm settled DEAE–Sephacel matrix. 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 100 and 400 mM NaCl containing eluting buffers whereas *μ*–calpain was eluted with 200 mM NaCl. Fractions were stored at 4 °C until assayed.

Casein zymography analysis

Methodology as reported by Biswas, Kripriyalini, Tandon, Sharma, and Majumdar (2016) 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. The gel was pre–run for 15 min at 100 V, 4 °C with running buffer (pH 8.3) containing 192 mM glycine, 25 mM tris–base, 1 mM EGTA [ethylene glycol–O–O'–bis (2–aminoethyl)–tetra-acetic acid], 1 mM EDTA, and 0.05 % (v/v) MCE. The supernatant or crude extract and sample buffer (3:1, v/v) containing 150 mM tris–base (pH 6.8), 20 % glycerol, 0.75 % 2–mercaptoethanol and 0.04 % bromophenol blue was mixed and loaded into casein gel. Gel electrophoresis was performed at 100 V, 4 º C for 4 h. The gels were then incubated in proteolysis buffer (pH 7.4) comprising 20 mM tris–base, 10 mM ditheothreitol (DTT) at 4

mM concentrations of $CaCl_2$ for 18–24 h at 27±2 °C. The gel was then stained with Coomassie brilliant blue (CBB) staining solution for 30 min and then destained with frequent changes of destaining solution till the bands become clear. Gel with clear discrete calpains bands was analyzed with the help of white light illuminator and it was photographed.

SDS-PAGE analysis

As calpastatin remains undetectable in casein gel due to lack of proteolytic domain, but their identification is important to assess inhibition activity of this enzyme to calpains. For identification of calpains and calpastatin in different purified fractions, SDS–PAGE was performed. SDS–PAGE analysis was carried out as per the protocol described by Laemmli (1970) with slight modifications. The gels were cast in Mini–Protean® Tetra Gel System (Bio–Rad, India). The separating gels (10 %) and stacking (5 %) were prepared as per standard protocol. Purified fractions of calpains (*μ*-and-*m)* and calpastatin were mixed separately with sample loading buffer (1:2, v/v) containing 66 mM tris–base (pH 6.8), 26 % (v/v) glycerol, 2.1 % (w/v) SDS, 0.01 % (w/v) bromophenol blue and 5 % (v/v) MCE. The samples were heated at 95 °C for 5 min and then loaded into wells along with protein molecular weight marker.

The gel electrophoresis was done at 120 V, 4 ºC with the electrode running buffer containing 192 mM glycine, 25 mM tris–base (pH 8.3) and 0.1 % SDS till the tracking dye reached bottom of the separating gel. The gel was then stained with Coomassie brilliant blue (CBB) staining solution for 1–2 h, and then destained with frequent changes of destaining solution till the background of gel becomes clear. Gel with clear discrete protein bands was analyzed with the help of white light illuminator and it was photographed.

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, Goll, Zeece, Robson, and Reville (1976) 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 1N acetic acid), 5 mM $CaCl_2$, 1 mM NaN_3, 5 mg/ml casein and 10 mM 2–MCE. The reactions were incubated for 60 min at 25 ºC, and stopped by adding 2 ml of a 5 % trichloroacetic acid solution (TCA).

The denatured proteins were precipitated by centrifugation at 2000rpm for 30 min, and the soluble peptides were measured for absorbance at 278 nm using Biospectrometer (Eppendorf, Germany). For the determination of calcium–independent proteolytic activity of each fraction, $CaCl_2$ in the reaction mixture was replaced by 10 mM EDTA. To determine Ca^{++} dependent proteolytic activity, absorbance 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 mM NaCl and 400 mM NaCl in equilibration buffer for *μ*– and *m*–calpain containing fractions respectively in 1.5 ml of assay buffer containing either $CaCl_2$ or EDTA. 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.

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:

m–calpain pooled fraction, incubated with assay buffer containing $CaCl_2$

m–calpain fraction plus calpastatin fraction, incubated in assay buffer

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.

One unit of calpastatin activity was defined as the amount of calpastatin that inhibits one unit of *m*–calpain.

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

The pH of breast fillets was determined as per the methodology mentioned by Trout *et al.,* (1992). A Bench top digital pH meter (Eutech 2700) equipped with a glass electrode and automatic temperature sensors. Ten gram of sample was homogenized with 50 ml of distilled water for 1 min using pestle and mortar. The electrode was dipped into the suspension and the pH value of the sample was recorded.

Water holding capacity was determined by press method (Wardlow *et al.,* 1973). Two Whatman filter papers were weighed, 0.5 gram meat sample was weighed and this meat sample was pressed between the centres of two weighed filter papers. Polyethylene sheets or glass plates were kept below and above the filter papers with sample. Pressure was applied by keeping weights of 18.5 kg on it for 5 min. After 5 min weight was removed and the meat flake was weighed. Filter papers were dried at room temperature and weighed.

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, Ray, and Stiffler (1981) with slight modification. WBSFV of breast fillets was estimated by placing the cores of samples in the blade attached to the Warner-Bratzler shear force apparatus (Model 81031307, G.R. Elect. Mfg. Co. USA).

Frozen/ cooked sample cores of 1.5 cm^3 were used for estimating the shear force values. Ten observations were recorded for each sample to obtain the value of shear force in kg/cm^2.

Lovibond tintometer colour

The colour profile of breast fillets were measured using Lovibond tintometer (Model F, Greenwich, U.K.). The sample colour was matched by adjusting red (*a*) and yellow (*b*) units and the corresponding colour units were recorded.

The 'Hue' and 'Chroma' values were determined by using formula, $(\tan^{-1})$ b/a (Little, 1975) and $(a^2 + b^2)^{1/2}$ (Froehlich *et al.,* 1983) respectively, where a, red unit; b, yellow unit.

TBARS values

The extraction method described by Witte, Krause, and Bailey (1970) was used for determination of TBARS value with slight modifications. For this, 10 g of sample was triturated with 25 ml of pre–cooled 20 % TCA in 2M orthophosphoric acid solution for 2 min. The content was then transferred quantitatively into a beaker by rinsing with 25 ml of cold distilled water, well mixed and filtered through Whatman filter paper No. 1 (s. d. fine chemicals Ltd., Mumbai, India). Then 3 ml of TCA extract (filtrate) was mixed with 3 ml of 2–thiobarbituric acid (TBA) reagent (0.005 M) in test tubes and placed in a dark cabinet for 16 hrs.

A blank sample was made by mixing 3 ml of 10% TCA and 3 ml of 0.005 M TBA reagent. Absorbance (O.D.) was measured at fixed wavelength of 532 nm with a scanning range of 531 to 533 nm using UV–Vis spectrophotometer (Make– Varian). TBA value was calculated as mg malonaldehyde (MDA) per kg of sample by multiplying O.D. value with K factor of 5.2.

Free Fatty Acid (FFA) content

The method as described by Koniecko (1979) was followed for determination of free fatty acid (FFA) contents. For this, 5 g of sample was blended into fine powder using anhydrous sodium sulphate and then mixed with 30 ml of chloroform for 2 min.

The slurry was filtered through Whatman filter paper No. 1 into a 100 ml conical flask. About 2 – 3 drops of 0.2 % phenolphthalein indicator solution were added to the chloroform extract, which was then titrated against 0.1N alcoholic potassium hydroxide to get the pink colour end point. The quantity of potassium hydroxide required for titration was recorded and calculated as follows:

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

Peroxide value (PV)

The peroxide value was measured as per procedure described by Koniecko (1979) with slight modifications. Five gram of meat sample was blended with 30 ml chloroform for 2 min in the presence of anhydrous sodium sulphate. The mixture was filtered through Whatman filter paper No.1 and 25 ml aliquot of the filtrate was transferred to 250 ml conical flask to which 30 ml of glacial acetic acid and 2 ml of saturated potassium iodide solution were added and allowed to stand for 2 min with occasional shaking (swirling) after which 100 ml of distilled water and 2 ml of fresh 1 % starch solution were added. Flask contents were titrated immediately against 0.1N sodium thiosulphate till the end point was reached (non–aqueous layer turned to colourless). The peroxide value (meq/kg of the meat) was calculated as per the following formula:

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

Myofibrillar fragmentation index (MFI)

The myofibrillar fragmentation index (MFI) was determined in broiler breast fillet samples as described by Davis, Dutson, Smith, and Carpenter (1980) with slight modification. This basically measured the proportion of muscle fragments that passed through the muslin cloth after sample had been subjected to a high speed homogenization treatment.

Protein extractability

Salt extractable proteins (SEP) and water extractable proteins (WEP) were estimated as

per the method of Kang and Rice (1970). For water extractable proteins, 4 g of accurately weighed meat sample was homogenized with 30 ml of chilled distilled water in a pestle and mortar for 2 min and then transferred in to a 100 ml conical flask and kept overnight at 4 °C.

The slurry was centrifuged at 5000 rpm for 5 min and the supernatant was collected. The residue was re-extracted with 10 ml of chilled distilled water and centrifuged once again as above. Salt extractable proteins were estimated by homogenizing the residue (remaining after the extraction of water extractable proteins) with 30 ml of chilled 0.67 N NaCl for 2 min and left overnight at 4 °C.

The slurry was centrifuged in a refrigerated centrifuge at 5000 rpm for 5 min and the supernatant collected. Residue was re-extracted with 10 ml of 0.67 N NaCl, centrifuged and the supernatant was collected. The proteins extracted in 0.67 N sodium chloride solutions and plain distilled water was designated as salt extractable and water extractable proteins, respectively. The total proteins in these extractions were calculated by Bradford method.

Microbiological analysis

Standard plate count, Total coliforms count, Psychrotrophic count, *E. coli* count, *Staphylococcus* spp., *Salmonella* spp. and yeast and mould count (YandM) in the samples were enumerated as per the method described by American Public Health Association (APHA, 1984). Ready-made media (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) were used for enumeration of different microbes. Duplicate plates were prepared following pour plate method and the counts were expressed as $\log_{10}$ colony forming units (cfu) per gram of sample.

Statistical analysis

Experimental data were analysed statistically using standard software package as mentioned by Snedecor and Chochran (1994). 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 and 2). 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 24 h and were completely absent at 36 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, Tandon, and Sharma (2016) in breast muscle from White Leghorn. Similar findings were also reported by Huang, Huang, Ma, Xu, and Zhou (2012) in chicken breast muscle.

Identification of calpains and calpastatin in SDS-PAGE analysis

Results illustrated in Figure 3 and 4, 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 Figure 2 indicate that μ-calpain remained in its native form up to 24 h in post-mortem muscle sample and it was completely absent at 36 h. Table 1 showed that activity of μ-calpain was considerably higher just after slaughter and decreased to about 76 % at 36 h post-mortem. Similarly calpastatin activity decreased by 51 % at 36 h post-mortem, however *m*-calpain remained stable since its activity decreased by mere 19 % 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.,* (2016) for chicken breast muscle, however, the study conducted by Northcutt *et al.,* (1998) showed lower value of calpains fpr 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. 4). Assay analysis for calpastatin from breast muscle showed a decrease in activity by 27% whereas for thigh muscle it decreased by 31% (Table 1).

These changes in the activity of calpains and calpastatin may be due to decline of muscle pH during post-mortem aging (Table 1). Similar findings were also reported by Veiseth *et al.,* (2001) in beef and lamb muscles.

Changes in pH, Water Holding Capacity (WHC) and W-B Shear Force Value (WBSFV)

Results presented in Table 1 and Figure 5 observed that μ-calpain dependant postmortem aging of broiler breeder breast muscle was completed at 24 h and during that time pH of the sample was recorded to be 5.67, and thereafter, it decreased gradually. Various studies have revealed that breast muscle from chicken species developed post-mortem aging as early as 3 h or even after 24 h. This indicated that the post-mortem aging time relating to development of pH is poorly defined (El-Rammouz, Berri, Le Bihan, Babilé, and Fernandez, 2004; Veeramuthu and Sams, 1999). At slaughter, the mean pH values of the breast muscles were 5.94, and it gradually reduced up to 36 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 6 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 (Obanor *et al.,* 2005).

WHC of breast fillets were decreased ($P\leq0.05$) with the increase of ageing time, the value ranged from 24.43 % at 30 min post-slaughter to 7.67 % at 36 h during holding at (4±1) ºC. The WHC however decreased significantly ($P\leq0.05$) only after 18 h of refrigeration temperature ageing. 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.

Table.1 Effect of calpain mediated post-mortem ageing on quality of breast fillets during holding at refrigeration temperature (4 ± 1 °C)

Parameters[#]	Post-mortem ageing times (Hrs)						
	0.5	3	6	12	18	24	36
μ-calpain (Units/g)	0.89 ± 0.012^{g}	0.85 ± 0.003^{f}	0.81 ±0.008^{e}	0.63 ± 0.008^{d}	0.43 ±0.008^{c}	0.31 ± 0.005^{b}	0.22 ± 0.003^{a}
m-calpain (Units/g)	2.99 ±0.005^{g}	2.96 ± 0.005^{f}	2.93 ±0.006^{e}	2.87 ± 0.008^{d}	2.82 ±0.005^{c}	2.68 ± 0.003^{b}	2.41 ± 0.01^{a}
Calpastatin (Units/g)	1.83 ±0.006^{g}	1.77 ± 0.003^{f}	1.71 ±0.003^{e}	1.55 ± 0.006^{d}	1.38 ±0.003^{c}	1.12 ± 0.003^{b}	0.91 ± 0.01^{a}
pH	5.94 ± 0.06^{e}	5.86 ± 0.07^{d}	5.78 ± 0.04^{c}	5.72 ± 0.03^{bc}	5.69 ± 0.02^{b}	5.67 ± 0.04^{ab}	5.65 ± 0.10^{a}
WHC (%)	24.43 ± 0.67^{b}	22.33 ± 2.28^{b}	21.17 ±1.05^{b}	16.33 ±0.61^{b}	15.67 ±4.86^{b}	8.67 ± 0.99^{a}	7.67 ± 1.09^{a}
WBSFV (Kg/cm^{2})	3.70 ± 0.39^{c}	2.97 ± 0.58^{bc}	2.20 ± 0.53^{ab}	1.93 ± 0.44^{ab}	1.67 ± 0.42^{ab}	1.43 ± 0.41^{a}	1.07 ± 0.28^{a}
Redness (*a*-value)	1.12 ± 0.02^{b}	1.10 ± 0.10^{b}	1.08± 0.06^{b}	1.05 ± 0.02^{a}	1.05 ± 0.02^{a}	1.03 ± 0.02^{a}	1.03 ± 0.02^{a}
Yellowness (*b*-value)	1.03 ± 0.02^{a}	1.03 ± 0.02^{a}	1.05 ± 0.02^{a}	1.08 ± 0.02^{ab}	1.10 ± 0.04^{ab}	1.13 ± 0.05^{b}	1.15 ± 0.02^{b}
Hue angle	50.11 ± 0.46	50.76 ± 1.12	50.18 ± 0.84	50.90 ± 1.09	50.58 ± 2.08	50.86 ± 2.33	50.84 ± 0.52
Chroma	1.60 ± 0.02^{c}	1.58 ± 0.11^{b}	1.54 ± 0.05^{b}	1.51 ± 0.01^{a}	1.48 ± 0.02^{a}	1.46 ± 0.03^{a}	1.46 ± 0.03^{a}
TBARS (mg MDA/Kg)	0.31 ± 0.119	0.32 ± 0.002	0.33 ± 0.003	0.34 ± 0.001	0.36 ± 0.002	0.39 ± 0.006	0.42 ± 0.010
Peroxide value (meq/kg)	0.15 ± 0.02^{a}	0.15 ± 0.01^{a}	0.16 ± 0.02^{ab}	0.16 ± 0.02^{ab}	0.17 ± 0.02^{b}	0.17 ± 0.02^{c}	0.18 ± 0.01^{c}
FFA contents (%)	0.03 ± 0.001	0.04 ± 0.001	0.04 ± 0.001	0.05 ± 0.002	0.04 ± 0.001	0.05 ± 0.001	0.05 ± 0.003
MFI (%)	9.70 ± 0.36^{a}	12.82 ± 0.11^{b}	16.85 ± 0.78^{c}	22.98 ± 0.26^{d}	24.73 ± 0.34^{e}	26.22 ± 0.55^{f}	28.38 ± 1.54^{f}
WSP (%)	12.13 ± 0.31^{a}	13.13 ± 0.42^{ab}	13.74 ± 0.58^{abc}	13.98 ± 0.24^{bc}	14.26 ± 0.44^{bc}	15.07 ± 1.02^{c}	15.45 ± 0.70^{c}
SSP (%)	25.04 ±1.57^{d}	24.38 ±1.20^{d}	24.48 ± 1.24^{d}	21.42 ± 1.16^{c}	19.94 ± 1.09^{b}	18.38 ± 1.11^{a}	16.53 ± 1.08^{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 analysis of broiler breeder breast fillets at 0 hour and 24 hour aging at refrigeration temperature (4±1°C)

Parameters	T_1	T_2	P-Value
Standard plate count	3.10±0.02^{a}	3.18±0.02^{b}	0.011
Total coliform count	0.46±0.03^{a}	0.75±0.01^{b}	0.000
Psychrophilic plate count	1.44±0.02^{a}	1.74±0.01^{b}	0.000
E. coli count	0.23±0.01^{a}	0.55±0.01^{b}	0.000
Staphylococcus aureus spp. count	ND	ND	ND
Salmonella count	ND	ND	ND
Yeast and mould count	0.42±0.01^{a}	0.56±0.02^{b}	0.000

*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 24 h of ageing at refrigeration temperature ((4±1) °C)

Fig.1 Casein zymogram developed with non-calcium proteolytic buffer, Lane-1: 0 h breast muscle, Lane-2: 2 h breast muscle, Lane-3: 4 h breast muscle

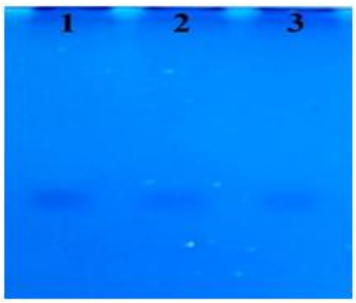


Fig.2 Casein zymogram shows clear bands of *µ*- and *m*-calpains of breast muscle from broiler breeder during ageing at refrigeration temperature (4 ± 1 ºC), Lane-1: 0 h breast muscle, Lane-2: 3 h breast muscle, Lane-3: 6 h breast muscle, Lane-4: 12 h breast muscle, Lane-5: 18 h breast muscle, Lane-6: 24 h breast muscle, Lane-7: 36 h breast muscle

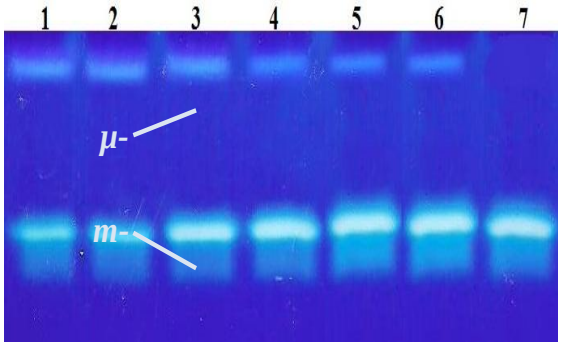


Fig.3 SDS-PAGE shows clear bands of calpains and calpastatin of breast muscle from broiler breeder immediately after slaughter (0 h), Lane-1 and 2: Calpastatin, Lane-3 and 4: *m*-calpain, Lane-5 and 6: *µ*-calpain [Protein Markers: Novagen Perfect Protein Markers (10 – 150 kDa)]

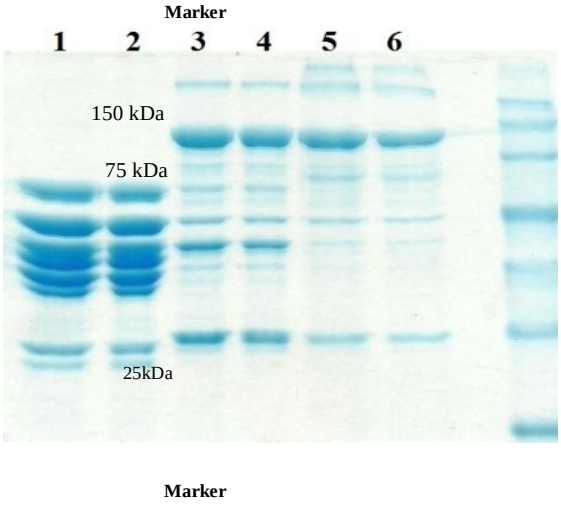


Fig.4 SDS-PAGE shows clear bands of calpains and calpastatin of breast muscle from broiler breeder 24 h of ageing at refrigeration temperature (4 ± 1 ºC), Lane-1 and 2: Calpastatin, Lane-3 and 4: *m*-calpain, Lane-5 and 6: μ-calpain [Protein Markers: Novagen Perfect Protein Markers (10 – 150 kDa)]

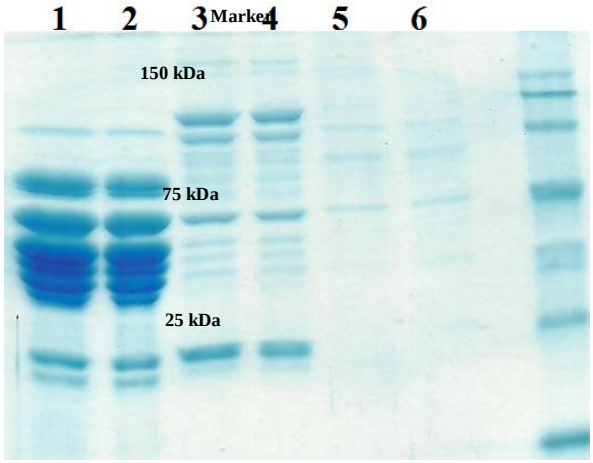
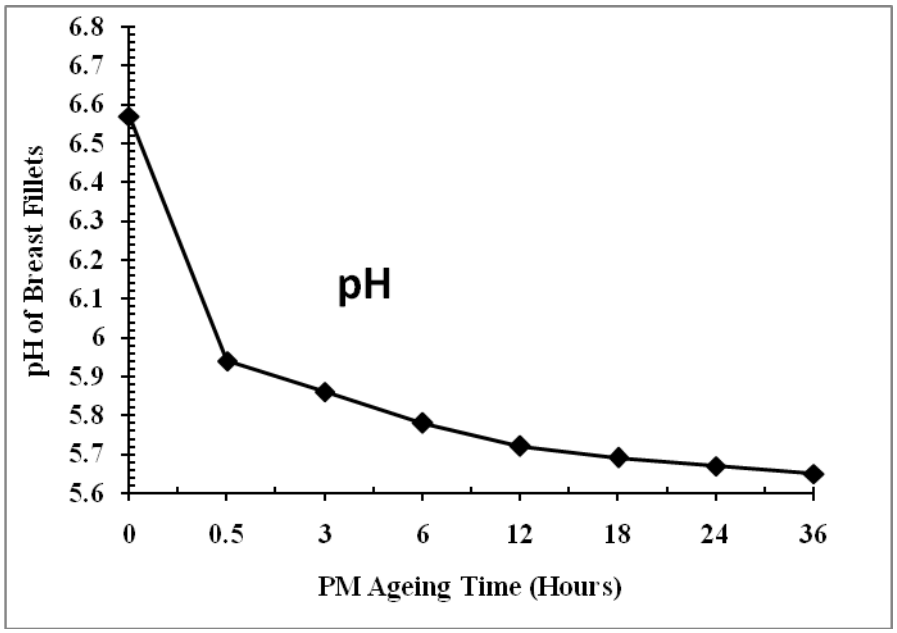


Fig.5 Changes in pH of chicken breast fillets during post-mortem ageing at refrigeration temperature (4 ± 1 ºC)



Similar to this study changes of WHC due to post-mortem ageing were reported by Mendiratta *et al.*, (2012).

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, Lyon, Windham, Lyon, and Savage (2004), 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. For the first 30 min-12 h of post-mortem ageing at (4±1) ºC, there was significant (P≤0.05) decreased in redness, as exhibited by *a*-values, but for 12 - 24 h *a*-value was decreased non-significantly. During initial 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.,* (1999). In comparison to *a*-value, *b*-value (yellowness) was little affected (in Table 1). The Hue angle (*H*) and Chroma (*C*) values were also followed similar trends to that of redness and yellowness value since they are estimated value. However, there was no definite trend observed for *H*, though Chroma values were decreased significantly up to 12 h for refrigerated ageing. As a whole, these results indicate that redness of breast meat colour change steadily during the first 0 – 12 h, while the carcasses are still in the processing plant. After 12 h, the colours continue to change but at a slower rate up to 12 - 24 h post-mortem.

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 0.31 mg MDA/kg at 30 min to 0.39 mg MDA/kg at 24 h during ageing of breast fillets shows that oxidative deterioration started from the initial period (30 min) and was increased until 24 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 (Botsoglou *et al.,* 2003). 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 as determines the fat status and quality of the muscle foods including broiler meat 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 differed significantly (P≤0.05) only after 6 and 12 h at refrigerated ageing (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. Similarly, significant increase of FFA contents in comminuted meat products were reported by Das *et al.,* (2008). Similar trends were found by Kripriyalini (2015) in turkey breast meat and by Biswas *et al.,* (2012) in chicken meat products. It was reported that increased levels of FFA had no toxicological effects and products of hydrolysis of fats/oils have no adverse effect on the nutritional quality of foods (Fritsch, 1981). So FFA determination alone did not provide guide for acceptability of the meats, but support as suitable quality indicator for oxidative changes of fat (Pearson, 1968).

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. The oxidative status of meat can be assessed in primary oxidation phase, by determining peroxide value. Post-mortem ageing at (4±1) ^{o}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 refrigeration temperature, and again these values were differed non-significantly (P≥0.05) amongst the different ageing periods (Table 1). At the end of ageing, the fillet sample in refrigerated ageing group was found around 0.17 meq/kg (Table 1). This result agreed with findings of Ramzija *et al.*, (2010) 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 (Kong *et al.*, 1986).

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 (4±1) ºC temperatures. The MFI was increased with ageing at refrigeration temperature (4±1) ºC until 24 h. Similar observations were also reported by Obanor *et al.*, (2005). When Kang *et al.*, (2012) studied the effect of artificial enzymes (bromelein and papain) on the myofibrillar fragmentation index; higher values were reported for treated samples than untreated ones. Similarly, breast fillets kept for ageing had higher MFI due to action of endogenous proteases (mainly μ-calpain) on muscle cells thereby improved meat tenderness than their counterpart meat samples that are not considered for post-mortem ageing.

Protein extractability (Water and salt soluble proteins)

The changes in protein extractability for water soluble proteins (WSP) and salt soluble proteins (SSP) as affected by post-mortem ageing at refrigeration temperature (4±1^{o}C) are presented in Table 1. It has been observed that with the ageing at refrigeration temperature WSP and SSP were increased and decreased (P≤0.05) respectively up to 24 h. 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 (2014) but a decrease in sarcoplasmic protein solubility with aging has been observed in beef (Bowker *et al.*, 2008). In pork, however, Boles, Parrish, Huiatt, and Robson (1992) observed day to day fluctuations in sarcoplasmic protein solubility but could not identify a clear trend with aging from 0 to 7 day post-mortem. 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 ($P \leq 0.05$) during storage between T_1 and T_2. Standard plate count was 3.08 and 3.18 log_{10} cfu/g at 0 and 24 hr ageing at 24 h (4±1) ^{o}C ageing. Total coliform count ranged from 0.46 and 0.75 at 0 hr and 24 hr ageing (4±1) ^{o}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 0.42 and 0.56 log_{10} cfu/g at 0 hr and 24 hr ageing at refrigeration 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 (Philips *et al.*, 2001). 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 (2005) reported high level of *Salmonella*, *Staphylococcus*, coliform, aerobic mesophiles in raw turkey meat samples. Significant ($P \leq 0.05$) 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.

Casein Zymography method was successfully employed for the simple and accurate identification of calpains in goat meat and blood samples. Casein Zymography method was successfully employed for the simple and 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 24 h during holding at (4±1) ^{o}C.

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