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Authentication of chilled and frozen chicken meat on the basis of mitochondrial enzymes

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ABSTRACT

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Global demand of quality meat and meat products is increasing day by day as fraudulent practices of meat are prevalent in meat market. Thus, correct labeling of meat as chilled/fresh and frozen-thawed is essential as consumer prefer chilled meat over previously frozen thawed meat due to its higher eating quality. Hence, authentication of chilled and frozen meat is necessary, and for this, enzyme based detection technique can be a great option as it is rapid, user friendly and cost effective. In this study, six samples of chicken meat were sets viz., (i) chilled meat (4±1ºC) (ii)meat kept for short term freezing at -20±2ºC for 7 days, (iii) meat frozen at -20±2 ºC without passing the rigor mortis, (iv) meat underwent single frozen thaw cycles (RFT1), (v) two times frozen thaw cycles (RFT2) and (vi) three times frozen thaw cycles (RFT3). Activity of four mitochondrial enzymes viz., β-hydroxyacyl coenzyme A dehydrogenase (HADH), citrate synthase (CS), aconitase (ACO2), aspartate amino transferase (AST) were assessed in meat express juice (MEJ) prepared from chilled and different treatments of frozen thawed meat. Results indicated higher ($P<0.05$) enzymatic activity in MEJ prepared from defrosted chicken meat than chilled meat and varied significantly ($P<0.05$) with each successive freeze thaw cycles. CS and aconitase has shown significantly ($P<0.05$) higher activities in MEJ prepared from frozen thawed chicken meat than other two mitochondrial enzymes. Thus, citrate synthase and ACO2 shown to have greater potential can be used in differentiation of chilled and frozen meat.
Keywords: Meat quality, Differentiation, Frozen Thawed Meat, Fresh Meat, Mitochondrial enzyme

INTRODUCTION

Now-a-days meat has become an integral component of the human diet because of its good nutritional values such as high quality protein, minerals, vitamins and essential fattyacids (Anzani *et al.*, 2020). In spite of that meat is considered as a highly perishable commodity, maintaining its quality for longer duration is very essential. Various microbes, chemical and oxidative reactions lead to meat spoilage and quality deterioration which eventually affect consumer acceptance (Biswas *et al.*, 2009; Lu *et al.*, 2022). Meat preservation can play a very important role during food scarcity, protects from microbial growth especially during long distance transportation of meat due to fluctuating temperature. Chilling and freezing of meat is a common practice to extend the shelf life of meat without alteration in nutritional quality (Biswas *et al.*, 2018). Chilling is done at 4°C for short term meat storage but for long term storage at commercial level most commonly employed method of meat preservation is freezing. Although there are pros as well as cons of freezing the meat such as traditional freezing leads to formation of large extra- and intracellular ice crystals, which ultimately causes muscle damage, loss of nutrients and in addition, protein deterioration and lipid oxidation also occur during long term frozen storage (Biswas *et al.*, 2013; Hou *et al.*, 2020). At the same time fraudulent practices of selling frozen meat of inferior quality as chilled meat are going on in meat market for a mere economic gain as consumers willingly pay high for chilled meat because of its superior quality over frozen thawed meat. Thus, correct labelling of meat as chilled meat and previously frozen-thawed meat before marketing is necessary as it ultimately affect consumer trust.

In the past, various techniques, spectroscopic (UV, FTIR, NIR, NMR),comet assay, electron microscopy, DNA based methods, Bio-imaging, sensory methods etc., have been used to differentiate fresh and frozen-thawed meat (Škorpilová *et al.*, 2019; Gallo *et al.*, 2018). All of these approaches have made a good progress and performance but still lacking in commercial application, as they are time-consuming, costly, multi-complex, requires modern instrumentation and need highly trained and skilled operators for analysis. Enzymatic methods can be a great option as user-friendly, cost effective, rapid and reliable method. At cellular level there are various catalytically active cytosolic and mitochondrial enzymes which releases in meat exudates during thawing of previously frozen meat. During traditional freezing the moisture present in meat converted into ice crystals which ultimately disturb the membrane ultra-structure, leads to solute concentration and alter cellular biochemical reaction which ultimately resulted in release of cellular and extracellular material from meat (Gottesman and Hamn,

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1984). Several studies (Šimoniová *et al.*, 2013, El-Hajj *et al.*, 2020, Škorpilová *et al.*, 2019 and Biswas *et al.*, 2023) have been conducted for determining various catalytically active mitochondrial and cytosolic enzymes activity such as β-hydroxyacyl coenzyme A dehydrogenase (HADH), aspartate amino transferase (AST), aconitase (ACO2), citrate synthase (CS) and lactate dehydrogenase (LDH) but their studies were limited to one or two specific enzymes. Thus, aim of the study was to screen activity of various mitochondrial enzymes in meat express juice prepared from chicken meat so that a robust mitochondrial enzyme (CS) based technique can be devised in near future to differentiate chilled from previously frozen thawed chicken meat.

MATERIALS AND METHODS

Chemicals and reagents required

Whatman filter papers (1001W grade), Acetyl CoA, S-acetoacetyl coenzyme A, Oxaloacetic acid, 5,5'-dithio-bis (2-nitrobenzoic) acid (DTNB), EDTA, β-Nicotinamide adenine dinucleotide phosphate (NADP), Isocitrate dehydrogenase, β-Nicotinamide adenine were procured from Sigma-Aldrich (Merck), USA. Tris-HCl buffer, potassium phosphate buffer ethanol, triton X100, acetic acid, buffer components, lactate dehydrogenase malate dehydrogenase, pyridoxal-5-phosphate, 2-oxoglutarate, L-aspartate, L-cysteine and other reagents used were of analytical grade and were procured from standard firms.

Sample collection

Meat samples of breast and thigh of approximately size of 100 grams each from freshly slaughtered chicken were procured from Experimental Poultry Dressing Plant of ICAR-CARI, Izatnagar and local meat shops of Bareilly. After collection the samples were transferred to lab in an ice box and visible fat, fascia and connective tissue were removed. The processed samples were packed in LDPE bags and subjected to further treatments.

Treatment of samples

The meat samples collected were divided into 4 groups- first group designated as chilled sample maintained 4±1°C, second group was passed through rigor development and then subjected to short term freezing at -20±2°C for seven days, third group was immediately subjected to freezing at -20°C ± 2°C for seven days (without development of rigor-mortis), and fourth group was also kept at -20±2°C but it was subjected to rigor development and sub-divided into three and given separate treatment- single freeze thaw cycles (RFT1), two time freezing-thawing cycles (RFT2) and three times freezing-thawing cycles (RFT3). For development of rigor-mortis and postmortem ageing meat samples are held at room temperature (27°C) All the meat samples (100 gm each) were properly packed into LDPE pouches. Every experiment was conducted thrice independently and duplicates sample were taken for each time and meat from different cuts was treated separately (Biswas *et al.*, 2008).

Preparations of meat express juice (MEJ)

For analysis MEJ was prepared by adding ten gram of meat pieces of one cm^3 size into ten ml of Tris HCl buffer (50 mM, pH 8.0) for 15 minutes at 4±1°C. MEJ was collected by filtration with Whatman filter paper (1001W grade) and centrifugation at 10000 rpm for 10 min at 4±1°C (Biswas *et al.*, 2023). The MEJ was analyzed on the same day of collection for the enzymatic activity.

Screening of mitochondrial enzymes in MEJ

A total of four mitochondrial enzymes were screened viz. Citrate synthase (CS), Aconitase (ACO2), β-hydroxyacyl coenzyme A dehydrogenase (HADH) and Aspartate transaminase (AST) by given methodology.

Citrate synthase (CS)

Citrate synthase enzyme activity was analyzed in MEJ by following the protocol given by Skorpilova et al. (2019) with suitable modifications. The activity was measured spectrophotometrically at 412 nm using a cuvette of 10 mm path length. An enzymatic reaction between acetyl CoA and oxaloacetate take place which is catalyzed by citrate synthase enzyme releases CoA-SH and on addition of 5,5'-dithio-bis (2-nitrobenzoic) acid (DTNB) a yellow colored product is formed which is measured spectrophotometrically. Ultimately the absorbance of TNB is measured in a cuvette of 10 mm path length at 412 nm by using Bio-spectrophotometer (Eppendorf, Germany). The reaction mixture contained 170 µl of reaction buffer (pH 8.0), 10 µl of MEJ, 5 µl of acetyl coenzyme A, 10 µl of oxaloacetic acid and 20 µl of DTNB in and the mixture was incubated for five minutes at room temperature. The CS activity was expressed in terms of micromoles of product formed per min by one ml of MEJ.

β-hydroxyacyl coenzyme A dehydrogenase (HADH)

The HADH activity was analyzed in MEJ by following the protocol given by Boerrigter-Eenling (2017) with suitable modifications. HADH is present in inner membrane of mitochondria and catalyzes the reaction in which acetoacetyl-CoA is converted to β-–hydroxyacyl–CoA and its presence is detected by taking the absorbance at 340 nm (Ballin and Lametsch, 2008). For analysis the reaction mixture consisted 100 ìl of 100 mM potassium phosphate buffer (pH 7.3), 10 ìl MEJ, 50 ìl 34.4 mM EDTA and 20 ìl of 7.5 Mm and incubated for 5 min. Then, 20 ìl S-acetoacetyl coenzyme A (5.9 mM) was added and absorbance was taken at 340 nm and the mixture further incubated for 4 min. and again absorbance was taken at 340 nm. The difference in absorbance before and after addition of S-acetoacetyl coenzyme A was taken and the activity of HADH was expressed as micromoles of product formed per min by one ml of MEJ.

Aconitase

The activity of aconitase enzyme was analyzed in MEJ prepared from chicken meat according to the method suggested by Skorpilova et al. (2014) with appropriate modifications. Aconitase is a Kreb's cycle enzyme, which converts citric acid into isocitrate by forming cis-aconitic acid as intermediate. Further isocitric acid is converted to á-ketoglutaric acid by oxidative decarboxylation and NADP is changed to NADPH. This increase in NADPH concentration is detected by taking the absorbance at 340 nm (Skorpilova *et al.*, 2014). For the measurement of activity 50 mM Tris HCl buffer (pH 7.4) with 1 mM EDTA, 10 ìl MEJ, freshly prepared 5 ìl cysteine (5 mM) and 2 ìl isocitrate dehydrogenase (2 mg/ml) were incubated for 10 min in a cuvette. The reaction was triggered with 5 ìl of 10 mM citrate and 10 ìl of 0.5 mM NADP and again incubated at 37æ%C for 15 minutes and later absorbance was taken at 340 nm by using Bio-spectrophotometer (Eppendorf, Germany). Further the ACO2 activity was calculated as micromoles of product per min per ml of meat express juice by applying Beer Lamberts law. The molar absorption coefficient of NADPH was 6220 M^{-1} cm^{-1}.

Aspartate transaminase (AST)

Meat express juice prepared from chicken meat samples were investigated for the presence of AST enzyme according to the method suggested by Adeyemi *et al.* (2015) with appropriate modifications. The reaction mixture contained Tris-HCl buffer (80 mM, pH 7.8), 5 ìl of lactate dehydrogenase (1 mg/ml), 5 ìl of malate dehydrogenase (1 mg/ml), NADH (180 ìM), pyridoxal-5-phosphate (100 ìM), 2-oxoglutarate (12 mM) and L-aspartate (240 mM). The reaction mix was incubated at 30 ! for 10 min without addition of 2-oxoglutarate. After 10 min reaction was triggered with the addition of 2-oxoglutarate and the change in absorbance was observed at 340 nm up to 3min by using Bio-spectrophotometer (Eppendorf, Germany) with a cuvette of 10 mm path length. Further the AST activity was calculated in terms of micromoles of product formed per min by one ml of meat express juice by applying Beer Lamberts law. The molar absorption coefficient of NADPH was taken as 6220 M^{-1} cm^{-1} (Biswas *et al.*, 2023).

Statistical analysis

Data for the statistical analysis were collected from the experiment conducted three times independently and duplicate samples were taken for each parameter. The results were expressed as the mean of three replicates (n=6) together with the standard deviation by applying one-way ANOVA using 'IBM-SPSS-Statistics-28.0' software packages. Student t-test was conducted for comparison of these data. Data between breast and thigh samples in the respective treatment groups were analyzed by two-way ANOVA and they were compared by DMRT test. The significance of data was expressed at $P < 0.05$.

RESULTS AND DISCUSSION

Results of activity data of citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase (HADH) are depicted in Table 1. The meat express juice collected from frozen thawed chicken meat stored at -20±2 ºC showed significantly ($P<0.05$) higher enzymatic activity than chilled meat. The possible reason behind this higher enzyme activity could be ice crystal induced damage to the cell membranes of organelles and stress induced by oxidation under freezing. Freezing affects the membrane ultra-structure property and concentrate the solutes in the meat, which alters the cellular biochemical reactions (Zhang *et al.*, 2017). Mitochondrial citrate synthase (CS) exhibited activity of 0.38±0.03 Units in MEJ prepared from chilled meat which was significantly ($P<0.05$) lower than frozen-thawed meat. The CS activity in further treatments of frozen thawed chicken meat increased significantly ($P<0.05$) with repeated freeze thaw cycles. The values of CS activity were nearly similar in MEJ prepared after short term freezing (STF) of meat and first frozen thaw cycle (RFT1). The CS activity was significantly ($P<0.05$) higher in MEJ prepared from meat which underwent second (RFT2) and third frozen thaw cycles (RFT3) than STF and RFT1. This might be due to higher pressure on cellular membranes by larger ice crystal formed during repeated freezing-thawing leads to release of more enzymes into extracellular spaces (Tippala *et al.*, 2021). The highest CS activity obtained in MEJ prepared from meat which undergone freezing at -20±2 ºC without passing rigor mortis (FTWRM) as more damage occur in the muscles due to severe form of rigor mortis sets in during thawing of the muscle (Yu *et al.*, 2005; Biswas *et al.*, 2020). These findings were in coordination with the results reported by Simoniova *et al.* (2013) for chicken meat. Although there was insignificant ($P>0.05$) difference observed in CS activity between MEJ prepared from breast and thigh cuts of chicken.

Aconitase is a Kreb's cycle enzyme whose activity is determined by increase in NADPH concentration which is detected by measuring the absorbance at 340 nm by using spectrophotometer. In this study MEJ prepared from chilled meat also shown aconitase activity of 2.40±0.11units but it was significantly ($P<0.05$) lower than the frozen thawed meat. Aconitase activity of chilled meat may be due to continuous post mortal changes and microbial activity (Skorpilova *et al.* 2014). The activity was increased significantly ($P<0.05$) during each repeated freeze thaw cycle which possibly due to recrystallisation leading to change in the ice crystals size and causing more muscle damage which results in more release of enzymes from intracellular spaces (Biswas *et al.*, 2016). There was insignificant ($P>0.05$) difference observed in aconitase activity between MEJ prepared from breast and thigh cuts of chicken meat. These findings were in

Table 1: Citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase HADH enzyme activity* in different chicken meat treatments

Treatments	CS		HADH	
	Breast	Thigh	Breast	Thigh
Chilled	0.38 ± 0.03^{dA}	0.39 ± 0.03^{dA}	0.27 ± 0.03^{cA}	0.23 ± 0.03^{cA}
STF	2.16 ± 0.08^{cA}	2.11 ± 0.05^{cA}	1.59 ± 0.20^{bB}	1.50 ± 0.08^{bB}
FTWRM	2.86 ± 0.05^{Aa}	2.77 ± 0.04^{aA}	2.01 ± 0.08^{abB}	2.07 ± 0.06^{aB}
RFT1	2.21 ± 0.08^{cA}	2.12 ± 0.07^{cAB}	1.83 ± 0.09^{abB}	1.74 ± 0.10^{abB}
RFT2	2.52 ± 0.10^{bA}	2.45 ± 0.05^{bA}	1.86 ± 0.05^{abB}	1.82 ± 0.07^{abB}
RFT3	2.70 ± 0.04^{abA}	2.61 ± 0.04^{abA}	2.17 ± 0.08^{aB}	2.02 ± 0.10^{aB}

n= 6; Mean±S.E. with different superscript column-wise (small letter)and row-wise (capital letter) differ significantly (P<0.05). STF= short term freezing; RFT=repeated freezing-thawing; FTWRM=freezing-thawing without completion of rigor mortis.
*Units: µmoles of product formed per min / ml of MEJ

Table 2: Aconitase and AST enzyme activity in different chicken meat treatments

Treatments	Aconitase		AST	
	Breast	Thigh	Breast	Thigh
Chilled	2.40 ± 0.11^{dA}	2.30 ± 0.07^{dA}	0.28 ± 0.03^{cB}	0.27 ± 0.03^{bB}
STF	5.76 ± 0.10^{cA}	5.82 ± 0.13^{bcA}	0.43 ± 0.03^{bB}	0.40 ± 0.03^{cB}
FTWRM	7.03 ± 0.06^{aA}	6.95 ± 0.07^{aA}	0.50 ± 0.03^{abB}	0.45 ± 0.04^{bcB}
RFT1	5.71 ± 0.08^{cA}	5.60 ± 0.10^{cA}	0.45 ± 0.01^{aB}	0.41 ± 0.02^{cB}
RFT2	6.23 ± 0.07^{bA}	6.17 ± 0.09^{bA}	0.60 ± 0.02^{aB}	0.55 ± 0.01^{abB}
RFT3	6.74 ± 0.07^{aA}	6.60 ± 0.07^{aA}	0.64 ± 0.02^{aB}	0.56 ± 0.01^{aB}

n = 6; Mean ± S.E. with different superscript column-wise differ significantly (P < 0.05). STF= short term freezing; RFT=repeated freezing-thawing; FTWRM=freezing-thawing without completion of rigor mortis.*Units: µmoles of product formed per min / ml of MEJ

coordination with the results reported by Skorpilova *et al.* (2014) and Biswas *et al.* (2023).

The enzyme aspartate transaminase (AST) was significantly (P<0.05) lower in chilled meat samples but the pattern of enzyme activities in different treatments of frozen thawed meat was different and values of AST activity were significantly (P<0.05) lower than other three enzymes as represented in Table 2. The AST values varied insignificantly (P>0.05) between different chicken meat cuts. The results were in coordination with the findings reported by Biswas *et al.* (2023).

CONCLUSIONS

The activity of mitochondrial enzymes in the frozen thawed chicken meat was significantly (P<0.05) higher than the chilled meat. Repeated freezing-thawing cycles leads to higher degree of tissue damage which ultimately resulted in swift increase of the mitochondrial enzyme activity. The finding of the present study revealed that mitochondrial enzymes especially CS and ACO2 could be acted as good biomarkers for the discrimination of chilled from frozen meat and further studied can be conducted for the development of rapid and sensitive enzymatic kit for field monitoring of meat supply chain.

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