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Rohit Kumar Jaiswal
Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar, India

Sanjod Kumar Mendiratta
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Jay Kishan Prasad
Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar, India

Suman Talukder
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Sagar Chand
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Gowtham Prasad Bomminayuni
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Vandita Mishra
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Corresponding Author
Rohit Kumar Jaiswal
Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar, India

Differentiation of fresh from frozen thawed buffalo meat by citrate synthase enzymatic assay

Rohit Kumar Jaiswal, Sanjod Kumar Mendiratta, Jay Kishan Prasad, Suman Talukder, Sagar Chand, Gowtham Prasad Bomminayuni and Vandita Mishra

Abstract
Buffalo meat has gained popularity due to beneficial nutritional characteristics such as reduced fat, cholesterol and calories and higher units of essential amino acids, biological value and iron content. These led to an exceptional increase in demand for fresh meat among consumers. This instigates some retailers to sell frozen-thawed meat as fresh which is having higher demand. Considering this, the study was undertaken for differentiation of fresh from repeated frozen-thawed buffalo meat by measuring activities of different intra-cellular mitochondrial enzymes. Buffalo meat was collected, packed aerobically in LDPE pouches and subjected to different freeze-thaw cycles to simulate the fraudulent practices prevailing under field conditions. Meat press juice collected from fresh as well as frozen-thawed meat after different cycles using compression method was subjected to citrate synthase enzymatic assays. The results of enzymatic assays revealed that activity of citrate synthase does not show significant difference among fresh, 1st freeze-thaw cycle and 2nd freeze-thaw cycle buffalo meat press juices. However, pH and WHC of buffalo meat were increased, whereas TBARS, thawing loss, cooking loss and aerobic plate count were increased during progress of freeze-thaw cycle for buffalo meat. Therefore, citrate synthase based enzymatic assay of meat press juice could not be used as effective method for differentiation of the fresh and repeated frozen-thawed buffalo meat.

Keywords: Enzymatic assay, citrate synthase, buffalo meat, frozen-thawed

Introduction
Among different categories of available meat, buffalo meat is in demand due to low fat and cholesterol content and no ban for slaughter by state and central government authorities. India has produced 1.58 million tonnes of buffalo meat for the year 2019-20 (BAHS, 2020) [1] and exported 1.08 million tonnes of buffalo meat products to the world for the worth of Rs. 23460.38 Crores during the year 2020-21 (APEDA, 2021) [2]. Increasing demand for buffalo meat and meat products indicates that it is highly relished by consumers at both national and global level. Indian consumer generally prefers fresh meat than frozen meat, due to their specific eating habits and preference of taste. Fresh meat when kept under improper storage conditions, are more prone to spoilage thereby affecting desired sensory attributes and microbial safety. Low temperature preservation techniques specially freezing are efficient storage methods for extending the shelf life of fresh meat. Therefore, retailers and consumers are relying on freezing fresh meat to extend shelf life. So, some retailers fraudulently mislabel the product and selling frozen-thawed meat as fresh meat (Simoniova *et al*., 2013) [3]. Due to regular reporting of unlawful selling frozen-thawed meat as fresh meat, and to prevent consumer interest and meat producer issue, it is essential to distinguish fresh from frozen-thawed meat (Ballin and Lametsch, 2008) [4]. In absence of awareness regarding an effective method to distinguish between fresh from frozen-thawed meat, this problems are generally overlooked.

Numerous methods based on different scientific principles such as sensory detection, comet assay, enzymatic assay, DNA based assay, visible and near infrared reflectance spectroscopy, nuclear magnetic resonance, bio-imaging and enzymatic methods have been developed to discriminate fresh and frozen-thawed meat (Ballin and Lametsch, 2008) [4]. Amidst these methods, enzymatic method has been acclaimed as a method of choice due to its swiftness and economics.

Among different mitochondrial enzymes, Citrate synthase is a typical intracellular enzyme released after freezing and satisfies the conditions for use as specific markers (Hamm and

Gottesmann, 1984) [5]. Enzymes released from the cracked mitochondrial membrane can be very well detected in exudate released during thawing of meat and nature of meat can be determined through the measurement of enzymatic activity in meat press juice. The concentration of enzyme in frozen-thawed meat press juice is considerably higher than in fresh meat, which further increases with progress of freeze-thaw cycle. It is possible to determine the activity of citrate synthase enzyme in a relatively small amount of meat press juice (5µl) (Simoniova *et al*., 2013) [3]. Therefore, till now no studies have been conducted for buffalo meat in respect to citrate synthase. The present study was done to develop rapid and reliable citrate synthase enzymatic assay to distinguish between fresh and frozen-thawed buffalo meat

Materials and Methods

Sampling, collection of meat press juice and estimation of citrate synthase activity

Halal slaughtered buffalo *longissimusdorsi* muscle was procured from local market of Bareilly. The samples were taken to the laboratory in insulated ice-box and packed in LDPE pouches and subjected to different treatments. At 8hrs of postmortem, each chilled muscle was cut into three pieces with 3.0 cm thickness, perpendicular to muscle fiber orientation. The first treatment was analyzed directly as the control (fresh); in second treatment, sample was frozen at -18±2 °C for 48hrs and thawed at refrigerated temperature (4±1 °C) for 12hrs (1st freeze-thaw cycle) and in third treatment, 1st freeze-thaw cycle sample was again re-frozen at -18±2 °C for 48hrs and thawed at refrigerated temperature (4±1 °C) for 12hrs (2nd freeze-thaw cycle). These treatments were carried out to simulate the conditions of fraudulent practices occurring under field conditions. Meat press juice from fresh and frozen-thawed meat after different cycles was collected using compression method (Cheung *et al*., 2015) [6]. The activity/concentration of citrate synthase enzyme was determined in buffalo meat press juice as per the method defined by Simoniova *et al*. (2013) [3] with suitable modifications.

Physico-chemical and microbiological parameters

Along with citrate synthase activity assay, the three treatments groups of samples were also evaluated for physicochemical, microbiological and colour characteristics to determine the effect of freeze-thaw cycles on quality of buffalo meat.

pH and TBARS (2-thio barbituric acid reactive substances) value

Buffalo meat pH value and TBARS were measured according to the method of Trout *et al*. (1992) [7] and Tarladgris *et al*. (1960) [8] respectively.

Water holding capacity (WHC)

WHC was calculated as weight loss percentage based on measurements before and after compression of meat and expressed as:

$$\text{WHC}\ (\%) = \text{T1-T2/ T1}X100$$

Where, T1 = Initial weight of meat before application of pressure; T2 = Final weight of meat after application of pressure.

Thawing loss

Thawing loss was calculated by method of weighing different categories of meat samples before freezing and after thawing.

$$\text{Thawing loss}\ (\%) = \text{T1} - \text{T2/T1 X 100}$$

Where, T1 = Meat sample initial weight before freezing; T2 = Meat sample final weight after thawing.

Cooking loss

5±1g of meat sample was weighed and packed in a heat-stable LDPE pouch and kept at 80 °C for 30 min in water bath. The surface of meat sample was dried and weighed and calculated as follows:

$$\text{Cooking loss}\ (\%) = [(W2 - W3) / W2] \times 100$$

Where, W2 = meat weight before cooking (g) and W3 = meat weight after cooking (g).

Aerobic Plate Count (APC)

APC of buffalo meat sample was determined using the APHA (2001) [9] method.

Statistical Analysis

The data generated for different parameters were compiled and analysed using SPSS (version 20.0 for Windows; SPSS, Chicago. 111. (U.S.A.). The data was be subjected to analysis of variance and Tukey's HSD tests for comparing the means to find the difference among groups. The smallest difference for two means was reported as significant different (P<0.05).

Results and Discussion

Citrate synthase activity in buffalo meat press juice for different freeze-thaw cycles

The mitochondrial enzyme citrate synthase involved in Krebs Cycle activity in meat press juice collected from fresh, 1st freeze-thaw cycle and 2nd freeze-thaw cycle buffalo meat were 0.0008, 0.0017 and 0.0018 mU/ml respectively (Figure 1). There was no significant difference in citrate synthase activity between fresh, 1st freeze-thaw cycle and 2nd freeze-thaw cycle buffalo meat samples. Contrary to this finding, increasing citrate synthase concentration during freezing-thawing in chevon and pork meat was reported by Gowtham *et al*. (2020) [10] and Pipek *et al*. (2014) [11] respectively. The enzyme activity rapidly increases in the exudates of thawed meat in all muscle parts of the carcass (Simoniova *et al*., 2013) [3]. Moreover, the citrate synthase activity even increases during the long-term storage of meat at temperature below freezing point, can change the inside nature of the cells, which in consequence, can damage the cells and the specific enzymes are released into the exudate. Therefore, a higher quantity of citrate synthase enzyme activity can be measured. Another reason for the increased activity of citrate synthase during the storage period at temperatures far below the freezing point could be damage to the cell organelles caused by the growth of ice crystals during the freezing process. Moreover, even during the storage period due to temperatures fluctuations, the ice crystals may grow or shrink, which damages the cell structure even more. Thus, more the damage to mitochondria, the higher the activity of citrate synthase (Simoniova *et al*., 2013) [3].

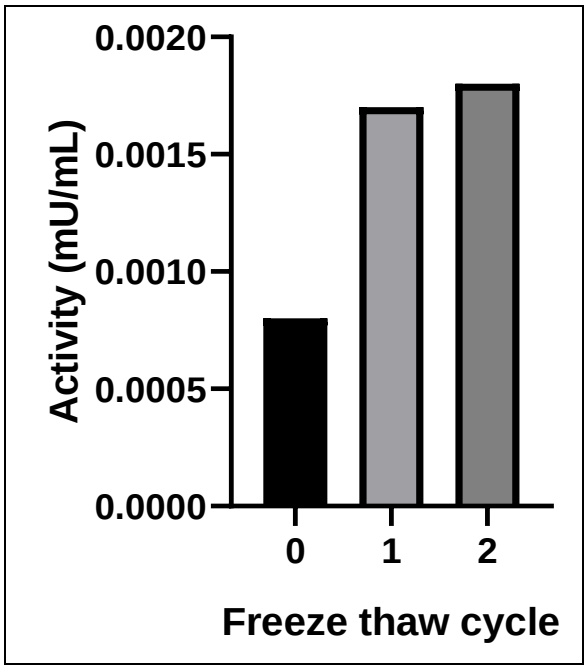

Fig 1: Citrate synthase enzyme activity in meat press juice of fresh, 1st freeze-thaw cycle and second freeze-thaw cycle buffalo meat

Changes in physico-chemical and microbiological parameters of buffalo meat for different freeze-thaw cycles

The pH of fresh buffalo meat sample was 6.29 which further decreased to 5.58 after second freeze-thaw cycle (Table 1). The significant difference (P<0.05) in pH among between fresh, 1st freeze-thaw cycle and second freeze-thaw cycle buffalo meat might be occurs due to continued conversion of glycogen to lactic acid which is responsible for lowering the pH. Frozen-thawed meat having lower pH than meat without freezing according to Leygonie *et al.* (2012) [12]. The pH of meat influences the rate of oxidative activity, microbial shelf life and water loss to a great extent (Rahman *et al.*, 2014) [13].

The TBARS value gives idea about the oxidative stability of meat during different freeze-thaw cycles. There were significant differences (P<0.05) reported among between fresh, 1st freeze-thaw cycle and second freeze-thaw cycle buffalo meat. The TBARS value reported for fresh, 1st freeze-thaw cycle and second freeze-thaw cycle buffalo meat were 0.11, 0.27 and 0.32 mg malonaldehyde /Kg of meat, respectively (Table 1). The TBARS trends obtained in this experiment was as par with TBARS of porcine *longissmusdorsi* (Xia *et al.*, 2009) [14] and beef *semimebranosus* (Cheng *et al.*, 2019) [15] muscle during multiple freeze-thaw cycles.

The effect of freezing and thawing on buffalo meat water holding capacity, cooking loss and thawing loss were also estimated to saw the overall effect on sensorial attribute particularly juiciness. The WHC reported for fresh, 1st freeze-thaw cycle and second freeze-thaw cycle buffalo meat were 68.93 56.11 and 53.88%, respectively which decreased significantly (P<0.05) with progression of freeze thaw cycles (Table 1). The thawing and cooking loss after one freeze-thaw cycle were 6.69 and 33.42 % respectively, and those would increase significantly (P<0.05) to 13.52 and 38.77 %, respectively after second freeze-thaw cycle (Table 1). From above finding, it is correlated that with increase in number of freeze-thaw cycles, WHC decreases with increase in thawing and cooking losses. Water loss during repeated freezing-thawing had major implications on meat product yield, appearance and overall it is related to WHC of meat (Huff-Lonergan and Lonergan, 2005) [16]. Repeated freezing-thawing causes melting and recrystallization phenomenon which lead to large size ice-crystals formation resulting in mechanical disruption of cell membranes and consequently loss of WHC (Srinivasan *et al.*, 1997) [17]. Similar trends of freeze-thaw effects on WHC, thawing and cooking loss have been reported by Srinivasan *et al.* (1997) [17] in shrimp muscle and Xia *et al.* (2009) [14] in porcine *longissimusdorsi* muscle.

Aerobic plate count values of buffalo meat samples increased significantly (P<0.05) with progress of freeze-thaw cycles. The APC for fresh, 1st freeze-thaw cycle and second freeze-thaw cycle buffalo meat were 3.74, 4.32 and 5.25 $\log_{10}$CFU/g respectively (Table 1). This might be due to cell membrane damage causing release of available nutrients to microbes causing its growth. Repeated freezing-thawing provide the time and favourable temperature where microbes can grow easily. The significant increased APC value recorded for freeze-thawed meat could be due to the leakage of fluid during thawing that is rich in nutrients required for microbial growth (Leygonie *et al.*, 2012) [12]. An increased APC values with increase in freeze-thaw cycles count was observed in chicken thigh muscle by Bae *et al.*, (2014) [18]. The result supports the findings of Katekhaye (2012) [19] for chevon who observed increased microbial load during different freeze-thaw cycles. In the present investigation, APC value of 2nd freeze-thaw samples was found to be >5 log cfu/g which is beyond the acceptable meat safety limit for fresh meat.

Table 1: Changes in physico-chemical and microbiological parameters of buffalo meat during freeze-thaw cycle

Physico-chemical parameters	Fresh meat	First freeze-Thaw cycle	Second freeze-Thaw cycle
pH	6.29±0.04 ^{a}	5.58±0.02 ^{c}	5.90±0.01 ^{b}
TBARS (mg/Kg)	0.11±0.01 ^{c}	0.27±0.02 ^{b}	0.32±0.03 ^{a}
Thawing loss (%)	ND	6.69±0.20 ^{b}	13.52±0.35 ^{a}
Cooking loss (%)	21.14±0.43^{c}	33.42±0.26 ^{b}	38.77±0.85 ^{a}
WHC (in %)	68.93±0.46^{a}	56.11±0.61^{b}	53.88±0.37^{c}
Aerobic Plate Count	3.74±0.10^{c}	4.32±0.12^{b}	5.25±0.04^{a}

N=6; Mean ± S.E. with different superscripts in row vary significantly (P<0.05), ND: Not detected

Conclusion

The activity of citrate synthase mitochondrial enzyme does not show significant difference among fresh, 1st freeze-thaw cycle and 2nd freeze-thaw cycle buffalo meat press juices. However, pH and WHC of buffalo meat were increased, whereas TBARS, thawing loss, cooking loss and aerobic plate count were increased during progress of freeze-thaw cycle for buffalo meat. The result suggests change in quality attributes of buffalo meat due to effect of various freeze-thaw cycle. The citrate synthase enzyme activity during various freeze-thaw cycle does not correlate significantly with changes in quality of buffalo meat. Therefore, citrate synthase enzyme activity assay could not be utilized for differentiation of fresh from frozen thawed buffalo meat.

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