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Differentiation of Fresh from Frozen Thawed Chevon by Citrate Synthase Enzymatic Assay

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A B S T R A C T

The objective of study was to differentiate fresh from frozen-thawed chevon by evaluating citrate synthase enzymatic assay and the effects of repeated freeze–thaw cycles (0–2) on physico-chemical and microbiological parameters in *longissimusdorsi* of chevon. The activity of themitochondrial enzyme in meat press juice from fresh and frozen-thawed meat was compared to know the efficacy of these enzymes in differentiating fresh from frozen-thawed meat. Citrate synthase activity had shown more significant change in values of meat press juice of fresh and frozen-thawed chevon. The physico-chemical and microbiological characteristics had also shown good correlation with the activity of enzyme at different stages of freeze-thaw cycles. A significant ($P<0.05$) reduction in pH, and water holding capacity; however, a significant increase in TBARS and thawing loss was observed from fresh to 2nd freeze-thaw cycle whereas total plate count showed increasing trend from fresh to 2nd freeze-thaw cycle. Therefore, citrate synthase enzymatic assay could be a suitable method for the differentiation of frozen-thawed from fresh chevon.

Introduction

Chevon is the most preferred and costliest meat sold in markets of northern India. The meat produced for the domestic market is mainly sold as fresh hot meat. Fresh meat is a perishable commodity with high water activity, unable to maintain its desired sensory and microbial attributes for a longer period of time at room temperature or inside chiller. So, unscrupulous retailers fraudulently mislabel their product, selling frozen-thawed meat as fresh meat for higher profit (Simoniova *et al.*, 2013). Due to increase in incidents of illegal selling of frozen-thawed meat as fresh meat, and in order to protect interest of consumers and domestic meat producers, it is necessary to differentiate fresh from frozen-thawed meat

(Ballin and Lametsch, 2008).In scarcity of information regarding an effective assay to differentiate between fresh and frozen-thawed meat, this type of marketing or mislabeling generally unnoticed. Several number of methods were proposed based on different 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 suggested to differentiate fresh and frozen-thawed meat (Ballin and Lametsch, 2008). Amongst all these methods, enzymatic assay has been recommended as the method of choice due to its rapidity and economics.

Enzymes released by damage to cell organelles by ice crystals during repeated freezing and thawing can be used as potential biomarker to differentiate fresh and frozen-thawed meat. The enzyme chosen for enzymatic assay must fulfill three requirements *i.e.* it should be released by freezing and thawing but not by ageing of meat; its total activity should not be decreased markedly during storage of muscle either fresh or frozen and it should be easily detectable in the muscle-press juice (Hamm, 1979).Citrate synthase is a typical intracellular enzyme released after freezing and satisfies the conditions for use as specific markers (Hamm and Gottesmann, 1984).Enzymes released from the ruptured mitochondrial membrane can be detected in exudate released during thawing and nature of meat can then be ascertained through the measurement of enzymatic activity in exudate. The activity of enzyme in frozen-thawed meat exudatesis considerably higher than in exudate of fresh meat, which further enhances with each successive freeze-thaw cycle.It is possible to determine its activityof citrate synthase,a specific mitochondrial enzyme in a relatively small amount of exudate (5µl) (Simoniova *et al.,* 2013).Thus, only limited studies have been conducted and no reliable method is available especially for chevon. The present study was carried out to develop rapid and reliable method to distinguish between fresh and frozen-thawed chevon

Materials and Methods

Sampling

Chevon *longissimusdorsi* muscle was procured from Experimental Abattoir of Livestock Products Technology Division, ICAR-Indian Veterinary Research Institute, Izatnagar and local market of Bareilly. The samples were transported to the laboratory in insulated ice-box under controlled conditions and packed in LDPE pouches and subjected to different treatments as per requirement. At 8hrs of postmortem, each chilled muscle was cut into three pieces with 3.0 cm thickness, perpendicular to muscle fiber orientation. The first sample was analyzed directly as the control (0 cycle). Other two pieces were frozen at -18±2^{o}C for 48hrs and these samples were thawed at refrigerated temperature (4±1^{o}C) for 12hrs, two cycles of freeze-thaw 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) (Fig. 1.). The activity/concentration of citrate synthase enzyme was determined in chevon meat press juice.

Collection of meat press juice

Meat press juice from fresh and frozen-thawed meat after different cycles was collected using compression method (Fig. 1.) as described by Cheung *et al.,* (2015). Meat cube was placed inside a plastic bag which was positioned between two Perspex plastic

plates attached with T-clamp screw. Meat press juice was obtained by exerting a pressure on the plastic bag for five minutes in case of fresh meat sample and two to three minutes for chilled or frozen-thawed samples. The activity/concentration of citrate synthase enzyme was determined in the meat press juice.

Citrate synthase (CS) enzymatic assay

Citrate synthase enzymatic activity was determined as per the method defined by Simoniova *et al.,* (2013) with suitable alterations. Meat press juice (0.5-1.0 ml) was collected from fresh and frozen-thawed chevon samples using compression method (Cheung *et al.,* 2015). The meat press juice was centrifuged (HERMLE Z32 HK, Germany)at 10,000 rpm for 5min at 4°C to collect the supernatant and remove insoluble material.5 µl of supernatant was added in to the wells and made the final volume of 50 µl using CS Assay buffer. 50 µl of the appropriate reaction mix (Table. 1) was added to each of the wells and mixed by pipetting and plate was incubated at 25°C.

The absorbance was measured at412nm using ELISA plate reader (BIO-RAD, iMark^{TM} Microplate Reader, Germany)at the initial time and taking measurements at every 5min for 20-40min while incubating at 25^{o}C. The final absorbance measurements for calculating the enzyme activity was the penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve. The time of the penultimate reading is T_{final}.

$$\text{CS Activity (milli unit/µl)} = \frac{S_a}{\text{Reaction Time x } S_v}$$

Where,

Sa = Amount of Glutathione (GSH) (nmole) generated in unknown sample well between T_{inital} and T_{final} from standard curve

Reaction Time= $T_{final} - T_{initial}$ (minutes)

Sv = sample volume (µl) added to well

Citrate synthase activity is reported as µmole/ml/min or milli unit/ml or mU/ml. One unit of citrate synthase is the amount of enzyme that generates 1.0 µmoles of CoA per minute at 25°C and pH 7.2.

Physico-chemical parameters

pH measurement

Muscle pH value was measured in triplicate according to the method of Trout *et al.,* (1992). Briefly, 5g of sample was homogenized (Ultra Turraxmodel T25, Janke and Kenkel, IKA, Germany)at 6000 rpm for 15s in 45 ml of distilled water (pH 7.0). The pH value of the homogenate was determined with a combined electrode connected to a Hanna pH meter (Hanna 211 Intruments, Italy).

TBARS (2-thio barbituric acid reactive substances) value

The distillation method of Tarlagdis *et al.,* (1960) was followed to estimate TBARS value.

Water holding capacity (WHC)

WHC was determined by the method of Li *et al.,* (2012) with slight modifications. Meat sample (2 g) was kept between two dried Whatman No. 1 filter papers (pre-weighed) and pressure of 343 N forces was applied for 5 min using a compression instrument (two Perspex plastic plates attached parallel with T-clamp screw, Germany). WHC was calculated as a percentage of weight loss based on measurements before and after

compression of meat and expressed as:

$$\text{WHC (\%)} = \frac{(W1)-(W2)}{W1} \times 100$$

Where,
W1 = Initial weight of sample before application of pressure
W2 = Final weight of sample after application of pressure

Thawing loss

Thaw loss was determined by method (AOAC, 1995) of weighing fresh, chilled, 1st freeze-thaw and 2nd freeze-thaw samples, before thawing and after thawing. Loss in terms of thawing of the meat samples was calculated by a percentage of weight loss of meat samples before and after thawing.

$$\text{Thawing loss (\%)} = \frac{(W1)-(W2)}{W1} \times 100$$

Where,
W1 = Initial weight of sample before thawing
W2 = Final weight of sample after thawing

Microbiological analysis

Total plate count (TPC)

The total plate count (TPC) was determined using plate count agar to evaluate the microbiological quality of fresh and frozen-thawed chevon meat samples.23.5 g of plate count agar was suspended in 1liter distilled water and boiled the medium to dissolve completely and sterilized the medium by autoclaving at 15 lb pressure at 121°C for 15 min. Final pH of medium was 7.0±0.2. Duplicate sets of petri dishes were inoculated aseptically with 1 ml aliquot from appropriate dilutions.

About 20 ml of plate count agar, melted and maintained at 44-46°C, was poured gently. The plates were incubated at 37±1°C for 48 h. Plates showing 30-300 colonies were selected for counting. The numbers of colonies counted were multiplied with reciprocal of the dilution and expressed the TPC count as $\log_{10}$cfu/g.

Results and Discussion

Citrate synthase (CS) activity

Citrate synthase activity value measured in exudate released from fresh, 1st freeze-thaw cycle and 2nd freeze-thaw cycle chevon samples were 0.034, 0.165 and 0.345µmol/ml/min respectively (Fig. 2). The activity of citrate synthase for fresh chevon samples ranged 0.034±0.003 µmol/ml/min, while 0.165±0.004 µmol/ml/min or more for the frozen-thawed chevon samples. The activity of citrate synthase was higher in exudate from frozen-thawed samples and increased with repeated freezing-thawing. The trend in activity of citrate synthase for chevon with subsequent freeze-thaw cycle was similar to the findings of Pipek *et al.*, (2010) in pork.

Citrate Synthase is mitochondrial specific enzyme to and it is possible to detect the enzymatic activity in a relatively less amount of meat exudate (5 µl) (Simoniova *et al.*, 2013). The increase in enzymatic activity is due to action of microorganisms present in the meat and ice crystals formed during freezing-thawing which might damage the muscle cells and mitochondrial membrane structure during thawing, and thus the endogenous enzymes could be released into the meat exudate during the storage. The increased activity of enzyme during the storage period at tem-peratures below freezing point of meat could be due to cell organelles damage caused by the growing of ice crystals during freezing-

thawing process. Furthermore, at storage period due to temperatures abuse and fluctuations, the ice crystals may grow or shrink subsequently damages the cell cytoskeleton. Thus, damage to mitochondrial membranes has positive and significant correlation with the activity of citrate synthase (Simoniova *et al.,* 2013).

Physico-chemical and microbiological parameters and its correlation with Citrate synthase activity

pH

The pH values (Mean±SE) of FM, 1FT and 2FT were 5.93±0.17,5.76±0.14 and 5.41±0.21 respectively (Table 2), which were decreased significantly ($P<0.05$) with the progression of freeze-thaw cycles. Muscle pH value decreased significantly ($P<0.05$) with increase in number of freeze–thaw cycles in chevon. It could be attributed to rapid decline of muscle temperature, and as a result, activities of glycolytic enzymes decreased accompanied by lowered rate of glycolysis (Savell *et al.,* 2005). Leygonie *et al.,* (2011) also reported that the pH of meat that has been frozen and thawed tends to be lower than prior to freezing. As pH is a measure of the amount of free hydrogen ions (H+) in a solution, it is possible that freezing with subsequent exudate production could cause denaturation of buffer proteins, the release of hydrogen ions and a subsequent decrease in pH (Leygonie *et al.,* 2011). This corroborates with the findings of Qi *et al.,* (2012) for ovine *longissmusdorsi* muscle, Ali *et al.,* (2015) and Chen *et al.,* (2017) for chicken breast muscle and Cheng *et al.,* (2019) for beef *semimembranosus* muscle.

TBARS

TBARS value determines the stability of lipid in meat. Available lipid in meat gets oxidized during storage. There was an increase in TBARS value (Mean±SE) of chevon from 0.19±0.02 mg MDA/kg in fresh sample, 0.36±0.05mg MDA/kg in 1stfreeze-thaw cycle, 0.69±0.03 mg MDA/kg in 2nd freeze-thaw cycle (Table 2). The lipid oxidation was found to be pronounced during repeated freeze–thaw cycles (Xia *et al.,* 2009). It is always concomitant with the degradation of unsaturated fatty acids (Hernandez *et al.,* 1999).

However, lipolysis of phospholipids could occur during subsequent thaw–freeze storage (Saldanha and Bragagnolo, 2008) and result increase in free fatty acids. Lipolytic enzymes were shown to keep active during frozen storage (Hernandez *et al.,* 1999). It was noted freeze-thaw cycles played an essential role in accelerating the oxidation, as the ice crystals had potential to damage the cell structure and subsequent release of certain pro-oxidants *viz.*oxidative enzymes, haem iron and free radicals for lipid oxidation (Benjakul and Bauer, 2001; Leygonie *et al.,* 2012).

The TBARS observed in this study was comparable with the TBARS of porcine *longissmusdorsi* (Xia *et al.,* 2009), beef *semimebranosus* (Cheng *et al.,* 2019) and ovine *longissmusdorsi* (Qi *et al.,* 2012) muscle during multiple freeze-thaw cycles.

Water holding capacity (WHC)

Measured values (Mean±SE) of WHC in chevon was 54.19±1.76% in fresh sample, whereas 42.24±1.28% after one freeze–thaw cycle, and increased ($P<0.05$) to 31.68±1.91% after 2nd freeze–thaw cycle(Table 2). In general, frozen storage with successive freezing-thawing contributes significantly to reduction in the water holding capacity of meat (Vieira *et al.,* 2009). The increase in water loss volume indicated loss of water-holding capacity of the muscle.

Table.1 Reaction mix

Reagent	Samples (µl)	Blank
CS Assay buffer	43 µl	45 µl
CS Developer	5 µl	5 µl
CS Substrate Mix	2 µl	-

Table.2 Meat quality parameters of chevon *longissimusdorsi* muscle samples derived from different freeze-thaw cycles (Mean±S.E.)

Parameter	Fresh meat	1st Freeze-thaw cycle	2nd Freeze-thaw cycle
Physico-chemical parameters			
pH	5.93±0.17^{a}	5.76±0.14^{b}	5.41±0.21^{c}
TBARS (mg MDA/kg)	0.19±0.02^{a}	0.36±0.05^{b}	0.69±0.03^{c}
WHC (%)	54.19±1.76^{a}	42.24±1.28^{b}	31.68±1.91^{c}
Thawing loss (%)	0	11.15±0.62^{a}	24.31±0.84^{b}
Microbiological parameter (log_{10}cfu/g)			
Total Plate Count	4.82±0.17^{a}	5.61±0.22^{b}	6.91±0.13^{c}

n=6; Values with different superscripts (a, b, c) in the same column are significantly different (P<0.05)

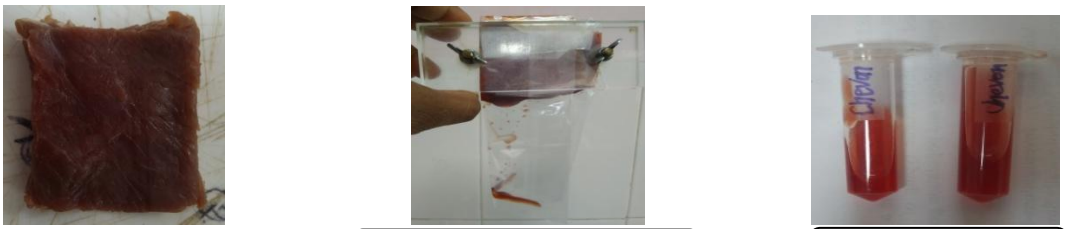

Fig.1 Collection of meat press juice from Chevon (Cheung *et al.*, 2015)

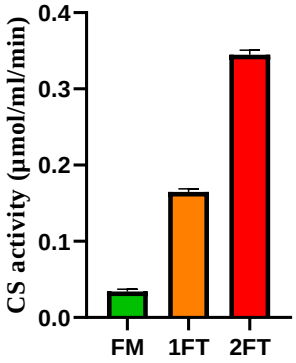

Fig.2 Citrate synthase activity (µmol/ml/min) values of FM (Fresh meat), 1FT (1st Freeze-thaw cycle) and 2FT (2nd Freeze-thaw cycle) of chevon

This might be due to mechanical damage to cell membranes by repeated melting and reformation of ice crystals during freezing and thawing in multiple freeze–thaw cycles (Aroeira *et al.,* 2016). It weakens the protein matrix (protein oxidation) leading to increased toughness, loss in myofibrillar protein solubility (Benjakul and Bauer, 2000) and loss of water-binding capacity during repeated freezing and thawing. In addition, lipid oxidation stimulates changes in protein structures and in this manner manipulates WHC of the muscle (Davies, 2005). However, this phenomenon could be explained by a high rate of proteolysis, protein oxidation and the lower pH during repeated freeze–thaw cycles.

Thawing loss

The thawing loss (Mean±SE) of chevon was 11.15±0.62% after one freeze–thaw cycle, and increased (P<0.05) to 24.31±0.84% after 2nd freeze–thaw cycle. The relatively high value of thawing loss might be due to the results of freeze denaturation, in which both water in the form of drip and other substances leak from the damaged cells during the process of freeze-thawing (Burgaard and Jorgensen, 2011). The majority of the water loss as drip contributed from the immobilized water in the myofibrillar proteins of the meat (Duun and Rustad, 2008). The findings of the present study were close to the values reported by Leygonie *et al.,* (2012) in Ostrich M. *iliofibularis*, Katekhaye *et al.,* (2012) in chevon *longissmusdorsi*, Xia *et al.,* (2012) in porcine *longissmusdorsi* and Cai *et al.,* (2014) in Japanese sea bass. All of them noticed increase in thawing loss with increase in number of freeze-thaw cycle.

Total plate count (TPC)

Total plate count values of chevon meat samples increased significantly (P<0.05) during repeated freezing and thawing. This might be due to cell damage, causing release of nutrients, making them more available to bacteria. Repeated freezing-thawing increases the time that the meat is at a temperature where bacteria can grow easily. The TPC values (Mean±SE) of chevon in fresh, 1st FT and 2nd FT were 4.82±0.17,5.61±0.22 and6.19±0.13logcfu/g (Table 2).The significant increased (P<0.05) TPC 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).

An increased TPC values with increase in freeze-thaw cycles count was observed in chicken thigh muscle by Bae *et al.,* (2014). The result supports the findings of Katekhaye *et al.,* (2012) for chevon who observed increased microbial load during different freeze-thaw cycles. In the present investigation, TPC value of all the samples was found to be (ranging from 4.82 to 6.19 logcfu/g) within the acceptable meat safety limit (less than 7 log cfu/g). Although the degree of freshness or spoilage of meat is often evaluated by plate counts, it is known that spoilage is not the result of bacterial count per se but is caused by enzymatic activity and biochemical changes brought about by the growing microflora.

Citrate synthase activity increased during repeated freeze-thaw cycles of chevon and proved to be a suitable marker enzyme for the detection of temperature abuse during freezing and differentiation of fresh from frozen-thawed meat. Meat pH and WHC were decreased; however TBARS and thawing loss were increased: whereas, total plate count was increased with increase in number of freeze-thaw cycle. Increase in enzymatic activity was found well correlated with undesirable changes in meat quality during repeated freezing and thawing.

Therefore, citrate synthase enzymatic assay could be used as biomarker to differentiate fresh from frozen-thawed chevon.

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