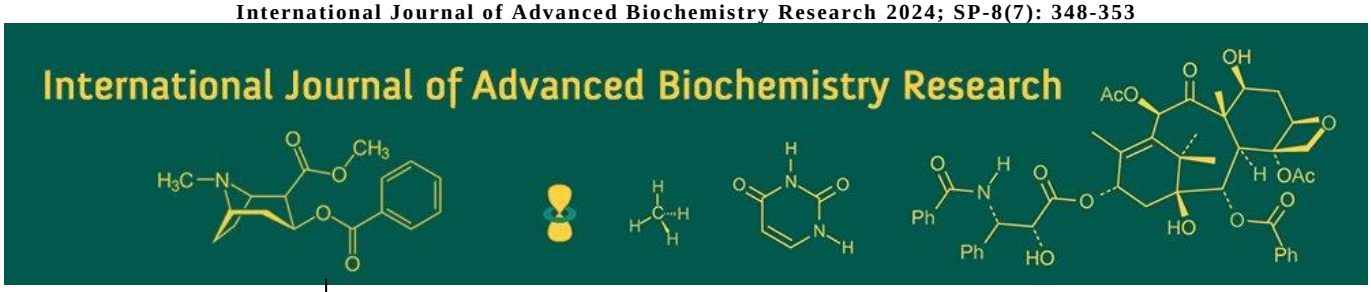

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Identification of superoxide dismutase as a novel biomarker for the differentiation of fresh and frozen-thawed goat meat

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Abstract
Labeling defrosted meat as fresh is a prevalent fraudulent practice in the meat supply chain. Among various analytical techniques for distinguishing between fresh and frozen-thawed meat, enzyme biomarker-based methods are crucial and reliable. This study identifies superoxide dismutase (SOD) as a new biomarker enzyme for differentiating fresh and frozen-thawed goat meat. A fourfold increase in SOD enzyme activity was observed in frozen-thawed goat meat due to muscle injury from freezing and thawing. Meat express juice (MEJ) was prepared by dipping method to minimize false positives. SDS-PAGE analysis of MEJ showed thicker bands for frozen-thawed samples, indicating higher protein content of MEJ from the frozen-thawed sample. DEAE Sephacel ion-exchange chromatography was employed to purify MEJ and fractions with high protein concentrations were analysed by SDS-PAGE and dot blot assay to confirm the presence of SOD. This study establishes SOD as a reliable biomarker for distinguishing fresh from frozen-thawed goat meat.

Keywords: Superoxide dismutase, novel biomarker, frozen-thawed goat meat

Introduction
Meat and meat products are unavoidable components of most of the human population. Global meat production and consumption have skyrocketed in recent years. The market share of the meat industry is expected to reach 1.3 trillion dollars by 2027 [1]. India has a diverse and voluminous population of livestock and poultry. According to the 20th National Livestock Census, a total head of 536.76 million livestock and 851.81 million poultry are reported in India [2]. The Basic Animal Husbandry Statistics of India for the year 2023 reported a total meat production of 9.77 million tonnes, positioning India as the world's 8th largest meat-producing country [3]. A constant growth rate of around 5 percent in meat production was observed during the last decade, except during the COVID-19 period. Along with the growth of the meat industry, there are multiple quality control issues arising in the meat sector day by day [4]. Food authentication in the context of meat and meat products is essential in addressing the growing problem of food fraud. This deceptive practice, often carried out by dishonest food businesses, involves inadvertently or even intentionally misleading consumers about the quality and contents of food, usually for economic gain [5]. In the meat industry, this can include falsely labelling poor-quality meats as premium cuts or adding fillers or substitutes. The meat and meat processing industry has witnessed numerous types of fraudulent practices so far [6]. One common practice is that it involves falsely labelling or misrepresenting the origin, quality, or content of meat products. The mislabelling of meat poses a significant concern in certain regions globally, particularly where consumers can distinguish the quality disparity between fresh and frozen-thawed meat. Unfortunately, a substantial number of consumers remain uninformed about this distinction and are consequently misled. In the European Union, the introduction of the Food Information to Consumer Regulations in 2014 marked a turning point, fostering greater consumer awareness regarding the disparities between 'fresh' and 'defrosted' meat. This regulation mandated the labeling of 'defrosted' for products, including meat, which underwent the frozen-thawed process.

However, such protective measures are notably absent in many densely populated developing countries. Studies reported that 8-15% of labeled fresh meat was frozen-thawed meat [7]. To protect consumer rights, there should be stringent measures to avoid the labeling of frozen-thawed meat as fresh meat. Otherwise, it is a complete violation of the Consumer Protection Act.

Myriad analytical methods are followed to differentiate fresh meat from frozen-thawed meat. A drastic change in the evolution of methods has been witnessed over the past years. Earlier methods were laborious and required complicated laboratory instrumentation and technical expertise. Currently, studies are going on to develop methods that need less intervention of experienced technicians and equipment. Methods available for differentiation of fresh and frozen-thawed meat can be broadly classified into sensory methods, quality analysis, microscopy (scanning electron microscopy) [8], enzymatic and electrophoretic methods [9], DNA-based methods, spectroscopy-based tests, dielectric properties-based tests, and mass spectrometry-based methods. Enzyme biomarker-based methods are one of the least explored areas for this purpose [10]. The enzymatic method detects cellular biomarker enzymes released due to freezing-induced cell injury, investigating enzymes from the cytoplasm, mitochondria, lysosome, and red blood cells [11]. Specific marker enzymes are identified for different species based on characteristic changes in muscle architecture. Identification of newer biomarkers for differentiation of fresh and frozen-thawed meat holds good potential in the meat quality control sector [11]. So far, various mitochondrial and lysosomal enzyme biomarkers have been explored for the purpose, such as Citrate synthase (CS) [12], β-hydroxyalkyl coenzyme A dehydrogenase (HADH) [13], Aconitase [14], ATP synthase, N-acetyl-β-glucosaminidase, Fumarase, lipoamide dehydrogenase, etc. Only citrate synthase enzyme has been explored as the biomarker enzyme for the differentiation of fresh and frozen-thawed goat meat, otherwise known as chevon [15]. The current study aims to identify and confirm a new biomarker enzyme for differentiating fresh goat meat from defrosted one.

Materials and Methods

Collection and processing of meat

Meat samples were collected from the experimental abattoir, Division of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar. About one-year-old male 'Rohilkhandi' breed goat (*Capra hircus*) was slaughtered as per scientific procedure, and tenderloin (*M. Psoas major*) was collected hygienically. About 100 g of each of the samples with different sets, was transferred to the laboratory and kept at a chiller (4±1 °C) to pass rigor. After passing rigor, chilled/fresh meat samples were prepared. One set of samples was kept at -20±2 °C for 7 days for freezing and thawing of meat was done at 4±1 °C for 12 hr. Drip was collected after complete thawing, and MEJ was prepared (RFT_1) as mentioned below. For the second freeze-thaw cycle (RFT_2) RFT_1 meat sample thawed completely was frozen again at -20±2 °C for 7 days and thawed at 4±1 °C for 12 hr. MEJ was prepared (RFT_2). For the third cycle, two times frozen-thawed samples were frozen again at -20±2 °C for 7 days, and after, thawing of meat was done at 4±1 °C for 12 hr. RFT_3 MEJ samples were prepared.

Preparation of meat express juice

Meat express juice (MEJ) was prepared by dipping method. About 10 gm of meat was cut into 1 cm^3 size and was dipped in 10 ml of 75 mM tris-HCl buffer at pH 7.6 for 15 minutes at 4±1 °C. After a dipping time, meat chunks were removed and the extract was filtered by using 1001W grade Whatman no.1 filter paper (Axiflow, India). The sample was prepared for both fresh and frozen-thawed goat meat samples and stored at -20±2 °C till further analysis.

Evaluation of difference in SOD of fresh and RFT MEJ

To check the difference in SOD levels at fresh and frozen-thawed samples, MEJ prepared from each sample was run in SDS-PAGE. The basic procedure of SDS-PAGE was adopted from Laemmlli (1970) [16]. The gel was prepared according to the requirements of the experiments. For resolving MEJ, 10% resolving gel and 4% stacking gel were prepared. Samples were treated with non-reducing dye to protect the dimeric molecular weight of the targeted enzyme. The first lane was loaded with molecular weight markers of molecular weight ranging from 5 to 245 kDa (BLUelf Prestained Protein Ladder, Simply, India). PAGE was run for 60 minutes at 100 V potential. After completion of the run, the gel was stained by 0.1% Coomassie Brilliant Blue and destaining was done. Bands were visualized in a gel documentation system.

Determination of SOD activity in the MEJ

The activity of the superoxide dismutase (SOD) enzyme was determined spectrophotometrically by employing the PMS (Phenazine Methosulfate)–NADH (Nicotinamide Adenine Dinucleotide Hydrogen)–NBT (Nitro Blue Tetrazolium) system using the protocol [17]. The reaction mixture consisted of 140 µl of 50 mM Tris-HCl (pH 8.0), 20 µl of NADH (205 µM), 15 µl of NBT (184 µM) and 15 µl of PMS (1.9 µM). For assaying, the SOD enzyme was pipette into a cuvette at room temperature (25 °C) containing freshly prepared NBT and NADH. The amount of formazan produced was proportional to the amount of superoxide radicals present, and therefore, to the activity of SOD. The assay was typically performed by incubating a sample of SOD with NBT, NADH, and PMS. The absorbance of the mixture was then measured at 560 nm, and the activity of SOD was calculated based on the amount of formazan produced. The reaction was initiated with the addition of freshly prepared PMS and the absorbance at 560 nm was continuously monitored as an index of NBT reduction using a UV-Vis spectrophotometer (Eppendorf Biospectrometer Basic, Germany). Reagent control was taken which was lacking the enzyme. The amount of product formed was calculated by Beer–Lambert's equation using the molar extinction coefficient for the PMS- NADH-NBT system as 25,000/M/cm (M^{-1} cm^{-1}).

Purification of SOD by Ion exchange chromatography

Aqueous ethanolic suspension of Diethylaminoethyl-Sephacel (DEAE-Sephacel) was procured from Sigma Life Science (Sweden) with an exclusion limit of 1000 kDa. For purification of protein from meat express juice (MEJ), 2200 µl MEJ and 800 µl drip samples were diluted in 2 ml 20 mM tris buffer of pH 8.0. The sample was loaded into a column by a pipette without disturbing the bed. The sample was drained out; collected in a centrifuge tube and reloaded again. The process was repeated three times. After the third

addition of samples, the setup was kept undisturbed for 1 hour. Washing was performed by adding 3 bed volume 20 mM tris-HCl. For every step of addition, it was taken care not to disturb the bed. Washout was collected and OD 280 was measured. Washing was done till OD_{280} became zero. Elution was performed with 10 ml of 75 mM NaCl and 0.5 ml of each eluate of 20 fractions were collected. OD_{280} of each fraction was determined and fractions having the highest OD were taken for further analysis.

SDS-PAGE analysis

SDS-PAGE analysis of each fraction having a higher OD was conducted. The basic procedure of SDS-PAGE was adopted from Laemmlli (1970) [16]. The gel was prepared according to the requirements of the experiments. For resolving MEJ, 10% resolving gel and 4% stacking gel were prepared. The first lane was loaded with molecular weight markers of molecular weight ranging from 5 to 245 kDa (BLUelf Prestained Protein Ladder, Simply, India). PAGE was run for 60 minutes at 100 V potential. After completion of the run, the gel was stained by 0.1% Coomassie Brilliant Blue and destaining was done. Bands were visualized in a gel documentation system.

Confirmation of SOD by dot blotting analysis

Confirmation of SOD in fractions was done by dot blotting analysis. A nitrocellulose membrane was cut at 2 x 2 cm dimension and the fraction having highest OD_{280} was loaded into NCM to make a dot in the membrane. For negative control, 10 mM PBS was used.

NCM was air-dried. Development of blot was done as per standard protocol. NCM was blocked by 10 ml blocking buffer (3% BSA) and incubated at room temperature for 2 hours. Blocking buffer was recovered and washing of NCM was done three times. The first two-time washing was done by TBST (tris buffered saline tween-20) and the third washing was done by TBS. Each washing was done for 10 minutes with mild agitation. After washing membrane was incubated with primary antibody (mouse anti-SOD monoclonal antibody) (SantaCruz Biotechnology, USA) overnight at 4 °C.

The primary antibody was prepared in 3% BSA (prepared in PBST) at a dilution of 1:500. After overnight incubation, washing was done as described before. The secondary antibody (anti-mouse-HRP) (SantaCruz Biotechnology, USA) was diluted to 1:2000 dilution in 3% BSA, added to the membrane, and incubated for 1 hour at room temperature. Washing was done as described before. The developing solution was prepared by 3 mg 3,3′-Diaminobenzidine (DAB), 4.5 µl hydrogen peroxide (H_2O_2), and 250 µl 3 M sodium acetate diluted in 14.75 µl double distilled water. A developing solution was added to NCM and allowed to develop colour. The reaction was stopped by the addition of double distilled water [18].

Results and Discussion

Evaluation of difference in SOD of fresh and RFT MEJ

The results of SDS-PAGE analysis of MEJ prepared from fresh and frozen-thawed goat meat samples showed a difference in thickness of bands at 32 kDa level, which is corresponding to the SOD enzyme's molecular weight. The results are represented in Figure 1. SOD is a dimeric enzyme having a molecular weight of 32.5 kDa, which is reflected in the results (9).

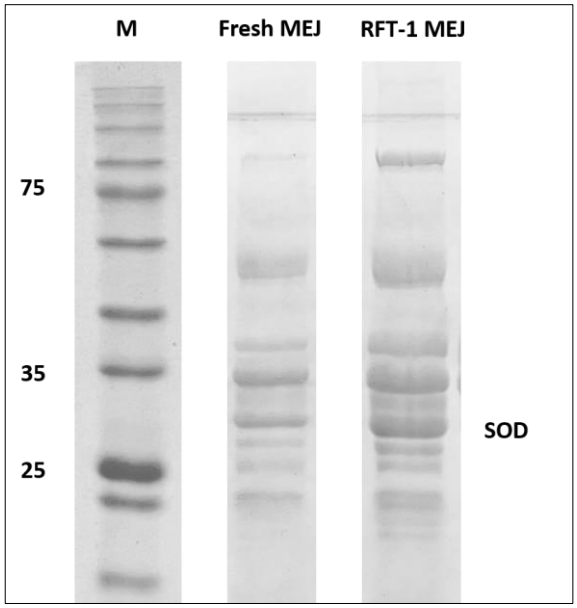

Fig 1: Results of analysis of MEJ from fresh and frozen-thawed (RFT_1) goat meat samples showing difference in band thickness. M- Marker protein

Determination of the activity of SOD enzyme in MEJ

The activity of the superoxide dismutase enzyme biomarker was analysed and the results are represented in Table 1. A significant difference (P<0.05) in the enzyme activity of chilled and frozen-thawed meat was observed. About four-fold increase in enzyme activity of SOD enzyme was observed for frozen-thawed meat (RFT_1) as compared to chilled meat. SOD enzyme level showed a non-significant decrease in its activity upon repeated freezing-thawing cycles of goat meat.

Meat that has been frozen and then thawed has mechanical damage to its organelles and muscle fiber structure. Damaged muscle fiber consequently discharges fluid into the extracellular space along with other cellular components. Thus, as a result of this occurrence, freeze-thaw loss or drip formation [19]. The formation of sharp ice crystals during freezing mechanically damages cell membranes, leading to the release of sarcoplasmic contents and associated enzymes. The SOD enzyme, primarily located in the cytoplasm, is released along with these contents, resulting in elevated levels in frozen-thawed meat (MEJ) [20]. Consequently, SOD activity is higher in frozen-thawed MEJ compared to chilled or fresh MEJ.

SOD is essential for neutralizing superoxide radicals, highly reactive molecules capable of causing significant oxidative damage to cellular structures. Freezing and thawing processes can compromise cellular integrity, leading to the production of reactive oxygen species (ROS), including superoxide radicals. This elevated oxidative stress activates SOD as a protective response. The variation in SOD activity is linked to ice crystal-induced damage during freezing. The formation of ice crystals in muscle tissues physically damages cell membranes and organelles, causing intracellular enzymes, including SOD, to leak into the meat express juice (MEJ). Additionally, freezing induces cellular stress and activates ROS production pathways, further enhancing SOD activity to counteract oxidative stress. This observation aligns with SOD's known role as the primary defense against cellular oxidative stress [20].

Therefore, the significantly higher SOD activity in frozen-thawed samples compared to chilled/fresh goat meat suggests its potential use as a biomarker for distinguishing between these two types of goat meat.

Table 1: Activity of SOD enzymes in MEJ extracted from fresh/chilled (4±1 °C) and frozen-thawed (-20±2 °C) goat meat samples

Enzyme activity	Chilled	RFT_1	RFT_2	RFT_3
SOD	2.87 ± 0.12^a	11.76 ± 0.42^b	11.57 ± 0.45^b	11.44 ± 0.38^b

Purification of SOD by ion exchange chromatography

MEJ was separated and purified using ion exchange chromatography. The results of the elution profile are represented in the figure 2. It was observed that the 14, 15, 16, and 17th fractions contain higher protein concentration as observed by higher OD 280 value. The meat express juice from frozen-thawed meat contains various proteins with different charges. Ion exchange chromatography was used to separate these proteins into different fractions based on their ionic charge. Anionic resins of DEAE (diethylaminoethyl) Sephacel, with a positively charged matrix, were utilized to separate negatively charged proteins. The results of anion exchange chromatography showed distinct peaks for different concentrations of elution buffers. A buffer concentration of 75 mM NaCl was selected for further analysis with SDS-PAGE. Based on the molecular weight of the SOD enzyme, which is 32.5 kDa, elution was anticipated at low to medium-low salt concentrations [21]. DEAE Sephacel chromatography is a type of anion exchange chromatography that separates molecules based on their charge. The DEAE groups, which are positively charged, are attached to a Sephacel matrix and attract negatively charged molecules such as the SOD enzyme. When a sample containing SOD is applied, the negatively charged enzyme binds to the positively charged DEAE groups. As the salt concentration in the elution buffer is gradually increased, the negatively charged SOD is displaced and eluted at specific salt concentrations (here it is 75 mM), allowing for its separation from other molecules based on their distinct ionic interactions with the DEAE resin.

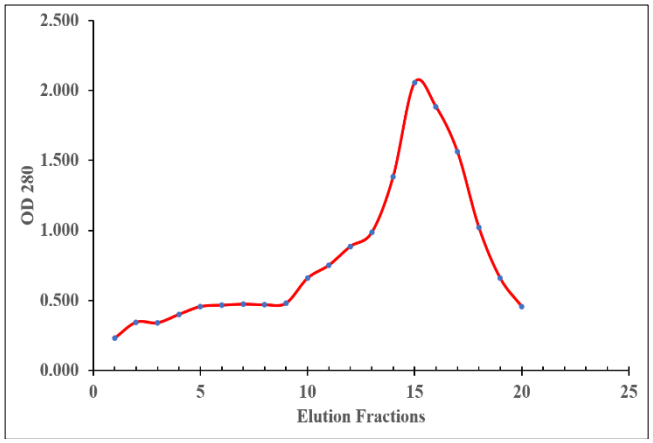

Fig 2: Elution profile of frozen-thawed MEJ at 75 mM NaCl concentration showing peaks at 14th to 17th fractions.

SDS-PAGE analysis of purified fractions

Results of SDS-PAGE analysis of fractions having the highest OD 280 showed specific bands between 25 to 35 kDa level as shown in figure 3. As discussed earlier, the dimeric configuration of the SOD enzyme was preserved due to the absence of a reducing agent such as β-mercaptoethanol in the sample dye (non-reducing sample dye) and it was reflected in the SDS-PAGE image. This distinctive band in the SDS-PAGE image provided concrete evidence for the successful isolation and purification of SOD from the meat express juice. The characteristic pattern observed in the image further confirmed the presence of SOD and its effective separation from other proteins in the sample.

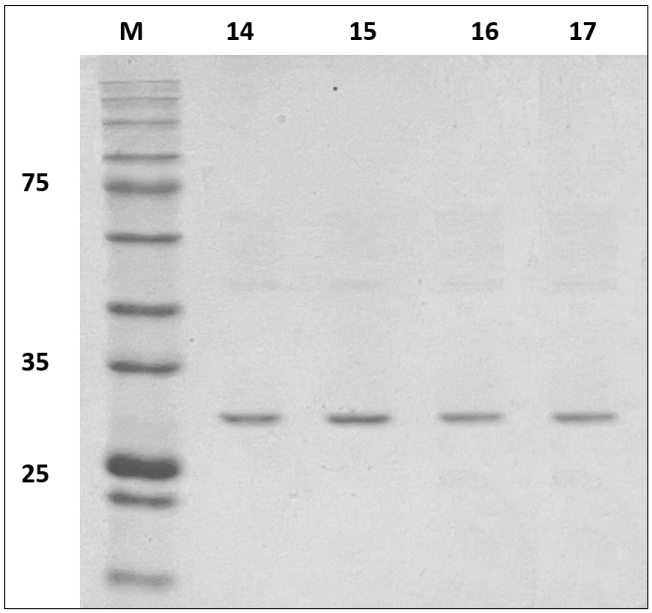

Fig 3: SDS-PAGE analysis showing the purified fractions 14, 15, 16, and 17 contain protein corresponding to the molecular weight of SOD (32.5 kDa).

Confirmation of SOD by Dot blotting

Results of dot blotting confirmed that the fractions separated from ion exchange chromatography were SOD enzymes. Figure 4 depicted the presence of a brown dot in the NCM where the fraction having the highest OD_{280} (fraction 15) was loaded. The presence of SOD enzyme in the fraction caused attachment of mouse anti-SOD monoclonal antibody and later HRP-conjugated anti-mouse antibody. Upon development, the brown-coloured precipitate was deposited in the site where the SOD enzyme was present causing the development of brown colour in the NCM. The results of dot blotting confirmed that the fractions purified by ion exchange chromatography, which was visualized at 32 kDa level in SDS-PAGE analysis were SOD enzymes.

The overall experimental finding suggested that fresh and frozen-thawed meat have significant (P<0.05) differences in SOD enzyme activity, which is proven in a sequence of experiments conducted here. This difference in activity suggested that cytoplasmic SOD can act as a reliable biomarker for differentiating fresh goat meat from frozen-thawed goat meat. The results of the current study found a four-fold difference which can be exploited for developing assays to differentiate fresh goat meat from frozen thawed goat meat. Further studies are required to expand the suitability of this biomarker in other types of meat too. Moreover, the development of assays can be explored using this novel biomarker enzyme to differentiate fresh and defrosted meat, which can be employed to prevent mislabelling food fraud. Development of such assays can

support the credibility of the meat supply chain as well as it can support the rights of consumers.

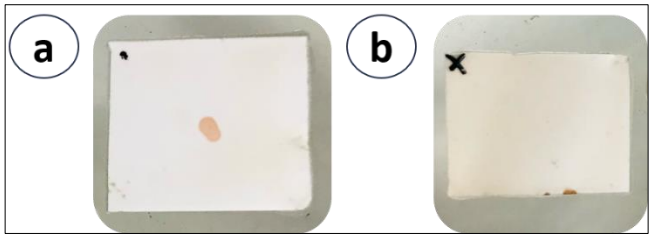

Fig 4: Results of dot blotting showing (a) the presence of brown precipitate in fraction 15 loaded NCM, confirming SOD in the purified fractions. (b) negative control showing no dot in the NCM.

Conclusions

The current study was undertaken to explore the suitability of cytoplasmic antioxidant enzyme, superoxide dismutase as a biomarker for differentiation of fresh and frozen thawed goat meat. The results of the study found that there is a fourfold difference in the activity of SOD enzyme in frozen-thawed goat meat as compared to fresh or chilled goat meat. This difference can be exploited for developing assays to differentiate both kinds of meat which can be employed in the meat quality control sector. The overall study concluded that the SOD enzyme can be an excellent biomarker for differentiating fresh goat meat from defrosted goat meat. Further studies are required to explore the suitability of biomarkers in other species origin meat as well as the development of suitable assays by exploitation of newly identified biomarker enzymes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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