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Research paper

Differential Expression of Myofibrillar Proteins during Chilling and Ageing of Chevon *Semitendinosus* Muscle

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ABSTRACT

Conversion of muscle to meat needs carcasses to be chilled for an extended period. During this process, several proteins are broken down into peptides and making meat more tender and palatable. Proteomic tools are employed to study these protein changes during ageing. Therefore, in this work, mass spectrometry was deployed to identify the differential expression of goat *semitendinosus* muscles proteins. Results revealed a total of 291 proteins of which 103 proteins were differentially expressed between aged and non-aged samples. In aged meat, up-regulated proteins were: myosin light chain/MLC-3 and MLC-1, myosin-I, myosin-II and myosin-III, troponin-C, histone, ATP synthase, cytochrome-C, annexin-A, creatine kinase and tropomyosin alpha chain. This study showed the advantages of ageing in goat meat and indicated probable protein markers associated with meat quality in goat meat.

Keywords*: Chevon, Proteomics, Ageing, Protein expression, Myosin light chain*

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INTRODUCTION

Chevon (goat meat) is most preferred and widely consumed meat in the country. Chevon contributes about 20% of total meat produced in India. In recent years, demand for goat meat is increasing due to rapid urbanization, increased income and demand for high protein food products. Currently consumer awareness on quality meat is increasing due to promotion of safe and hygiene meat by new enterprises and online platforms. However, the majority of meat marketed in domestic markets is still not chilled and aged and thus preventing conversion of muscle to meat. Post-mortem chilling and ageing of carcasses are primarily employed to ensure food safety, maximize shelf-life, reduce shrinkage and improve the tenderness of meat. There are many biochemical and structural changes that take place during this period of post mortem. In general, post-mortem degradation of several muscle proteins is an important factor in the meat tenderization process (Koohmaraie 1996; Hopkins and Thompson 2002). These changes in proteins are documented using proteomics which is recently being extensively applied in studying protein expression and degradation in meat and meat products. The proteome mapping investigation of bovine *semitendinosus* muscle using a combination of 2-DE and mass spectrometry carried out by Bouley *et al.* (2005) was one of the first steps towards understanding the mechanisms controlling post-mortem metabolism and meat quality. Previous studies on effect of chilling and ageing on expression individual skeletal muscles were focused on beef and lamb. But, similar studies in goat meat were not carried out.

Identification of differentially expressed proteins could be implemented as a molecular marker for goat meat quality. This may further provide new insights into the molecular mechanism of goat meat tenderness. Meat scientists around the world are looking for biological markers of tenderness to optimize the genetic selection of meat animals. Further, better understanding of proteomic changes that occur during post-mortem ageing is essential to develop strategies for improving fresh meat quality. Hence, to identify reliable proteins that control or determine goat meat quality especially tenderness, th[1]e present study was undertaken to compare expression of different muscle proteins between aged and non-aged goat meat.

MATERIALS AND METHODS

Animals, sampling, and treatments

Adult goat carcasses (Beetel breed; 12 months age) from animals slaughtered in government approved local municipality abattoir were used. A total of 12 goat carcasses (6 for each treatment) were randomly assigned to two post mortem treatments: 1. Control (4-6 h post mortem) and 2. Chilled and aged meat. Chilling was initiated at (16±1 ºC) within 2 h of post-mortem and continued for 6-8 h. For ageing fresh muscle samples of *semitendinosus* muscle obtained from chilled goat carcass were vacuum packaged in polyamide packaging films (Nylon-6, 100 µm) and stored in the chiller (6±2 ºC) for 72 h. Thus collected semitendinosus muscles were used for protein identification studies.

Protein identification with MALDI-TOF/TOF mass spectrometry from non-aged and chilled/aged meat sample

Salt soluble proteins (SSP) were extracted using the method of Kang and Rice (1970) with slight modification. 20 gram of minced meat sample was homogenized with 50 ml chilled 0.67 M NaCl in a polycarbonate centrifuged bottle for about one minute in Ultra Turrex T25 tissue homogenizer (IKA Labor Technik, Germany). Another 50 ml chilled 0.67 M NaCl was added and process was repeated. Volume was made to 200 ml with washings and sample was kept overnight in the refrigerator at 4±1 °C. It was then centrifuged at 3,000 g (Eltek Instrument, India) for 15 min. The fat layer was removed with the help of pre-sterilized spatula. The clean supernatant was used for protein identification studies. Proteomic service facilities of Centre for Cellular and Molecular Platforms (C-CAMP), Bangalore, India were utilized for the identifications of proteins using LC-MS/MS.

In-solution digestion and characterization of proteins using LC-MS/MS

Protein extracts were precipitated using ethanol and acetone (1:1), precipitated protein pellet was dissolved in 100 mM ammonium

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bicarbonate. Protein was quantified using BCA assay and approx. 50 µg protein was digested as per protocol (Wiese *et al.* 2007). All solvents and reagents used are of LC-MS quality. The 20 µg lyophilized enzyme was re-dissolved in 1.5 ml ice cold 1mM HCl (13ng/µl trypsin prepared) and 100 µl aliquots stored at -20°C and the trypsin vial was maintained on ice all throughout the preparation process. After thawing frozen aliquots, pH was adjusted by adding 15 µl of 50 mM ammonium bicarbonate prepared freshly. The pH of the protein sample was adjusted by adding 50 mM ammonium bicarbonate to ~8.5. Sample was vortexed and a short spin was given. Then, 13 ng/µl ice cold trypsin was added in 1:30 ratio and mixed well. Tubes with sample were placed into thermostat and incubated at 55 °C for 2 h or 37 °C for overnight. Tubes were then brought to room temperature and given a short spin. A 5% formic acid was added till the pH reached nearly 3 and mixed well before subjected to MS analysis.

Digested peptides were dried by speed vacuum and reconstituted in 100 µL of 2% acetonitrile with 0.1% formic acid and 1 µL of the same were injected on to the column. Digested peptides from in-sol samples were subjected to 70 LC run followed by acquisition of data on LTQ-Orbitrap-MS. Generated data was searched for the identity on MASCOT as search engine using Swiss-prot, TrEMBL and Capra Hircus databases. List of proteins and sequence generated through LC-MS/MS was used for the analysis.

LC-MS/MS analysis of differential protein expression in non-aged and aged meat

Extracted peptides were first desalted using ZipTip C18 (Millipore, U.S.A) protocols described by the manufacturer. The final elution volume following ZipTip clean-up was 1.5 µL. The peptide samples were then mixed (1:1) with matrix consisting of a saturated solution of CHCA (α-cyano-4-hydroxycinimic acid, Sigma) prepared in 50% ceric ammonium nitrite (CAN) /0.1% triflouro acetic acid (TFA). Aliquots of samples (0.6 µl) were spotted onto stainless-steel target plates. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (ABI 4800 plus, Applied Biosystems, USA) in the positive ion reflector mode. For precursor ion selection, all fractions were measured in single MS before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 20 most abundant precursor ion samples were selected for subsequent fragmentation by high energy CID. The collision energy was set to 1 keV and air was used in the collision gas. The criterion for precursor selection was minimum S/N of 0.5. The mass accuracy was within 50 ppm for the mass measurement and within 0.1 Da for CID experiments. The other parameters for searching were trypsin, 1 missed cleavage, variable modification of carbamidomethyl and oxidation of methionine, peptide charge of 1+, and monoisotopic. For database searches known contamination peaks such as keratin and auto proteolysis peaks for trypsin were removed before searching. Spectra were processed and analysed by the Global Protein server Explorer 3.6 software (Applied Biosystems, USA). This software uses an internal MASCOT (Matrix Science, UK) program for matching MS and MS/MS data against database information. The data obtained were screened against mammalian database downloaded from Swiss-Prot/TrEMBL homepage (http://www.expasy.ch.sprot).

RESULTS AND DISCUSSION

The gradual changes in temperature and pH during chilling are shown in Fig 1 and Fig 2. Temperature decreased to 15 °C within 4 hours of chilling. The time taken for carcass to reach the ultimate pH was about 6-7 hours. Bendall (1978) suggested that carcasses should be chilled slowly so that the internal temperature does not drop to less than 10 °C within first 10 h.

Fig. 1: Temperature profile of *semitendinosus* muscle of goat carcass during initial chilling at 16 ±1°C

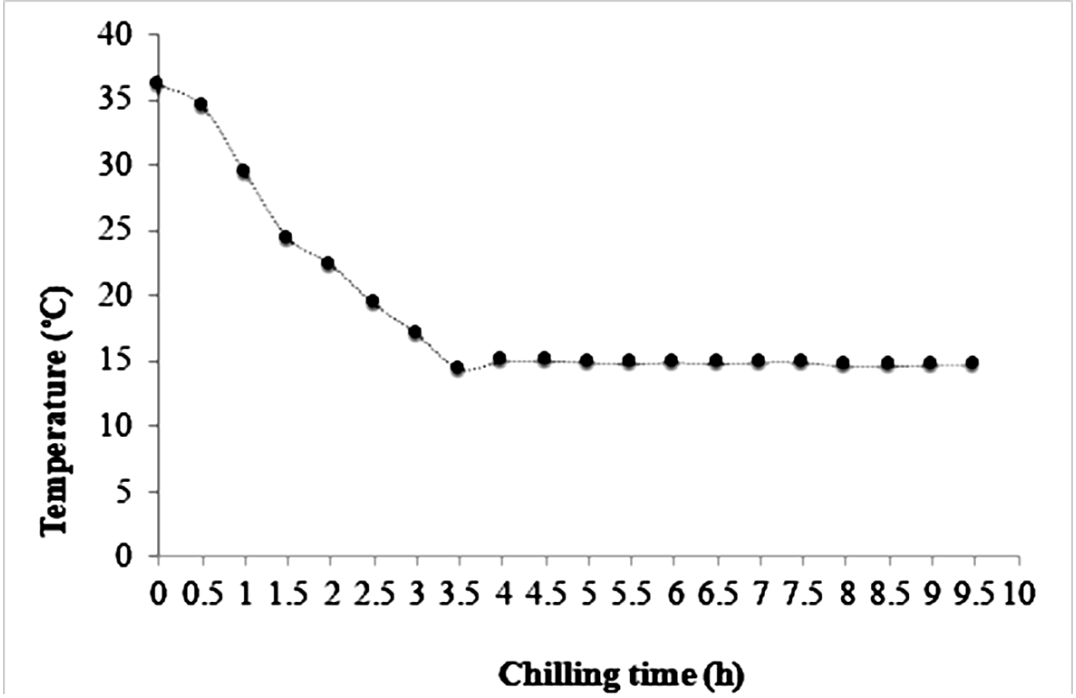
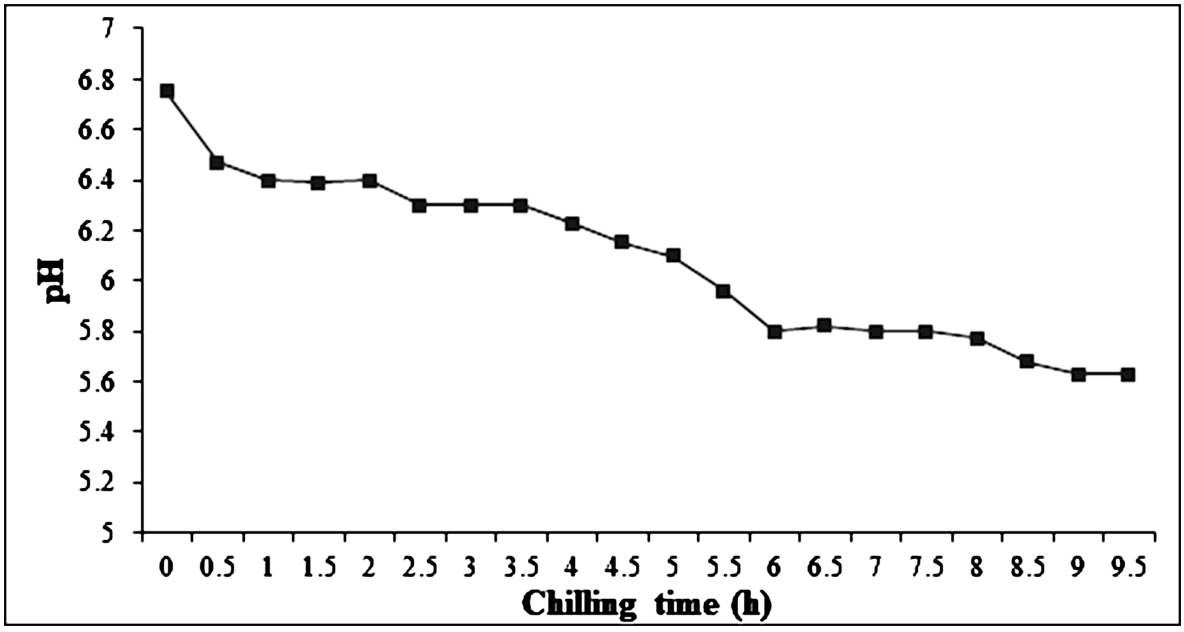


Fig. 2: pH profile of *semitendinosus* muscle of goat carcass during initial chilling at 16 ±1°C



LC-MS/MS identified myofibrillar, sarcoplasmic, mitochondria and ribosomal proteins. Myosin-1 was having highest scores and coverage among different myosin types. The score and coverage was higher for actin filament in aged/chilled meat. The titin identified in non-aged meat had higher score. Aged meat showed higher score for tropomyosin which provides physical integrity to muscle cells. Myosin light chain (MLC) found in aged meat had higher score. Major sarcoplasmic proteins identified are creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, 6-phosphofructokinase, enolase, and phosphoglucomutase). Endoplasmic reticulum calcium ATPase 1 was also observed in both the samples. HSP 70 identified in aged meat showed higher protein score and coverage. Other sarcoplasmic proteins identified were sarco/endoplasmic Calcium ATPase (SERCA) Calsequestrin, a calcium-binding protein of the sarcoplasmic reticulum and Calmodulin (CaM). Generally, tenderization of meat during ageing was attributed to various factors like sarcomere length, breakdown of proteins into smaller units (The proteolysis of troponin T occurs to a greater extent in meat with longer sarcomeres, noting that sarcomere length influenced Troponin T proteolysis in myofibrils (Weaver *et al.* 2009).

Table 1: Differential expression pattern of muscle proteins in aged *vs.* fresh goat meat

SNo	Accession	Protein name	Score	Sequence Coverage [%]	#Peptides	MW [kDa]	pI	Median (Aged: Fresh)
1	MYL3_BOVIN	Myosin light chain (Bos taurus)	220.1	21.1	4	21.9	5.0	6.79
2	ATPA_BOVIN	ATP synthase subunit alpha, mitochondrial (Bos taurus)	687.9	40.0	14	59.7	9.2	4.38
3	CYC_BOVIN	Cytochrome c (Bos taurus)	365.9	49.5	6	11.7	9.5	2.39
4	TPM3_BOVIN	Tropomyosin alpha-3 chain (Bos taurus)	660.40	38.4	12	32.8	4.7	1.57
5	KCRS_BOVIN	Creatine kinase S-type, mitochondrial (Bos taurus)	1054.9	49.9	17	47.2	8.5	1.46
6	MYG_SHEEP	Myoglobin (Ovis aries)	709.5	66.2	10	17.0	6.9	1.07
7	MYH1_BOVIN	Myosin-1 (Bos taurus)	8106.1	55.8	113	222.9	5.6	0.98
8	MYSS_RABIT	Myosin heavy chain, skeletal muscle	3033.9	33.0	38	125.4	5.1	0.96

9	TPM2_BOVIN	Tropomyosin beta chain (Bos taurus)	1026.9	47.9	16	32.8	4.7	0.94
10	MYH2_BOVIN	Myosin-2 (Bos taurus)	7052.2	47.1	97	223.2	5.6	0.94
11	MLRS_BOVIN	Myosin regulatory light chain 2, skeletal muscle isoform (Bos taurus)	583.1	62.9	10	19.0	4.9	0.81
12	TPM1_BOVIN	Tropomyosin alpha-1 chain (Bos taurus)	967.6	46.8	16	32.7	4.7	0.81
13	ACTB_BOVIN	Actin, cytoplasmic (Bos Taurus)	596.7	34.4	11	41.7	5.3	0.69
14	HSPB1_BOVIN	Heat shock protein beta-1 (Bos taurus)	150.3	30.3	5	22.4	6.0	0.67
15	MYH7_BOVIN	Myosin-7 (Bos taurus)	4026.3	41.9	74	223.1	5.6	0.56
16	ACTS_BOVIN	Actin, alpha skeletal muscle (Bos taurus)	1704.4	77.7	21	42.0	5.2	0.53
17	MLRV_BOVIN	Myosin regulatory light chain 2, (Bos taurus)	232.1	40.4	6	19.0	4.8	0.48
18	AT2A1_BOVIN	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (Bos taurus)	2366.8	40.1	33	109.2	5.2	0.34
	ACTN3_BOVIN	Alpha-actinin-3 (Bos Taurus)	2932.3	69.3	49	103.1	5.3	0.32
	ACTN2_BOVIN	Alpha-actinin-2 (Bos Taurus)	2550.50	66.3	46	103.7	5.3	0.17
	HS71A_BOVIN	Heat shock 70 kDa protein 1A (Bos taurus)	929.0	38.4	15	70.2	5.7	
	TNNT3_BOVIN	Troponin T, fast skeletal muscle (Bos Taurus)	331.0	22.1	6	32.1	6.0	

In beef, Anderson (2012) reported that myosin light chain 1 release from myofibrillar fraction during post-mortem ageing is a potential indicator of improved tenderness. In bull, *Longimuss dorsi* muscle, Wu *et al.* (2014) identified large proteins (>100 kDa) and their degradation products during post mortem ageing. The degradation of a few large structural proteins such as titin, nebulin has also been demonstrated to contribute to meat tenderness and other aspects of meat quality (Lonergan *et al.* 2010. Similarly, Farouk *et al.* (2012) identified different isoforms of myosin, myomesin, myosin, glycogen debranching enzyme and myosin binding protein C in beef.

Studies on differential expression of proteins indicated that chilling and ageing affected the protein expression in the muscle studied. Proteins expressed in different concentration in aged and non-aged meat were studied using peptide mass fingerprint and tandem mass spectrometry analysis and searching the results using Swissprot database. Overall about 291 proteins were identified using the database. Among 291, only 103 proteins were differentially expressed in aged and non-aged meat samples. Major up-regulated proteins in aged meat were: MLC 3 (6.8 folds), ATP synthase in mitochondria (6.7 folds), MLC 1(3.6 folds), troponin C (3 folds), cytochrome c, LDH (2 folds). Myosin-1, tropomyosin alpha chain, creatine kinase (1.5 folds). Major proteins down regulated in aged meat were: alpha actinin isoforms, SERC ATPase and its isoforms, pyruvate kinase, actin and creatine kinase. During the conversion of muscle to meat, major changes in muscle protein architecture are primarily noticeable at the expression levels of major myofibrillar proteins like myosin, actin, titin, nebulin, troponin-T, desmin and filamin (Lonergan *et al.* 2005). In a study by Jia *et al.* (2007) on protein changes in two different bovine muscles (*M. longissimus dorsi* and *semitendinosus*) after 24 h storage, five proteins (cofilin, lactoylglutathionelyase, substrate protein of mitochondrial ATP-dependent proteinase SP-22, HSP27 and HSP20) changed with a similar pattern in both muscles, while 15 proteins showed altered expression pattern specific for the two different muscle types. Stefania *et al.* (2010) observed changes in the insoluble protein fraction of bovine *longissimus thoracics* muscle from eight Norwegian Red (NRF) dual-purpose young bulls during the first 48 h post-mortem. They found significant changes in a total of 35 proteins and identified two metabolic enzymes (2, 3-bisphosphoglycerate mutase and NADH dehydrogenase) and one protein involved in the stress responses/apoptosis of the cell (Hsp70).

CONCLUSION

Ageing influences the protein degradation process and higher expression myofibrillar proteins was the hallmark of ageing process. Protein candidates like myosin light chain/MLC-3 and MLC-1, myosin-I, myosin-II and myosin-III, troponin-C, can be potential biomarkers for identifying good quality meat and suitable goat breeds for optimum meat production.

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ETHICS STATEMENT: Not Applicable

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