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Featured Article

Tandem mass tag labeling to assess proteome differences between intermediate and very tender beef steaks

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Abstract

Tenderness is considered as one of the most important quality attributes dictating consumers' overall satisfaction and future purchasing decisions of fresh beef. However, the ability to predict and manage tenderness has proven very challenging due to the numerous factors that contribute to variation in end-product tenderness. Proteomic profiling allows for global examination of differentially abundant proteins in the meat and can provide new insight into biological mechanisms related to meat tenderness. Hence, the objective of this study was to examine proteomic profiles of beef *longissimus lumborum* (**LL**) steaks varying in tenderness, with the intention to identify potential biomarkers related to tenderness. For this purpose, beef LL muscle samples were collected from 99 carcasses at 0 and 384 h postmortem. Based on Warner–Bratzler shear force values at 384 h, 16 samples with the highest (intermediate tender, **IT**) and lowest (very tender, **VT**) values were selected to be used for proteomic analysis in this study (n = 8 per category). Using tandem mass tag-based proteomics, a total of 876 proteins were identified, of which 51 proteins were differentially abundant ($P < 0.05$) between the tenderness categories and aging periods. The differentially identified proteins encompassed a wide array of biological processes related to muscle contraction, calcium signaling, metabolism, extracellular matrix organization, chaperone, and apoptosis. A greater ($P < 0.05$) relative abundance of proteins associated with carbohydrate metabolism and apoptosis, and a lower ($P < 0.05$) relative abundance of proteins involved in muscle contraction was observed in the VT steaks after aging compared with the IT steaks, suggesting that more proteolysis occurred in the VT steaks. This may be explained by the greater ($P < 0.05$) abundance of chaperonin and calcium-binding proteins in the IT steaks, which could have limited the extent of postmortem proteolysis in these steaks. In addition, a greater ($P < 0.05$) abundance of connective tissue proteins was also observed in the IT steaks, which likely contributed to the difference in tenderness due to added background toughness. The established proteomic database obtained in this study may provide a reference for future research regarding potential protein biomarkers that are associated with meat tenderness.

Lay Summary

Among all the eating quality attributes of beef, tenderness is considered an essential factor influencing consumers' overall satisfaction and future purchasing decisions. However, managing and predicting tenderness of meat products is challenging for the meat industry, as many factors can influence this attribute. The goal of this research was to examine variations in protein abundance between two categories of beef strip steaks varying in tenderness, with the intention to identify proteins related to beef tenderness/toughness. Overall, the results from this study suggest that tender steaks experienced greater protein degradation during aging than tougher steaks, which likely contributed to their improved tenderness. Furthermore, a greater abundance of connective tissue proteins, which are associated with meat toughness, was observed in the tougher steaks. Our results collectively indicate that the difference in tenderness between the two groups of steaks may be due to multiple proteins involved in several biological processes.

Key words: apoptosis, beef tenderness, metabolism, proteomics, tandem mass tag

Abbreviations: LL, longissimus lumborum; TMT, tandem mass tag; WBSF, Warner–Bratzler shear force

Introduction

Tenderness is regarded as one of the most important eating quality attributes of beef (Gagaoua et al., 2019), and research indicates that consumers are willing to pay up to 8% more for guaranteed tender beef (Alfnes et al., 2008). Conversely, variation in beef tenderness negatively influences consumer satisfaction as well as the future likelihood of repeat purchase (Van Wezemael et al., 2014). Therefore, the beef industry must provide products of consistent quality to meet consumer expectations and be perceived as high value. Despite years of investigation, however, we are still unable to precisely predict or control fresh beef tenderness. This is likely due, in part, to the complex nature and lack of understanding of factors contributing to variation in beef tenderness..

Skeletal muscle undergoes dramatic textural changes during the postmortem period that dictate end-product tenderness. These changes are mainly driven by two postmortem antagonistic processes, rigor mortis and proteolysis. While

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rigor mortis is responsible for muscle stiffness, proteolysis compromises the overall structural integrity of the muscle and is responsible for the improvement in tenderness during postmortem aging. Proteolysis in postmortem muscle involves the action of several endogenous proteolytic enzyme systems, including calpains, cathepsins, and caspases (Kemp et al., 2010). Yet, the calcium-dependent calpain system, more specifically calpain-1, has been widely regarded as the major protease responsible for postmortem proteolysis and tenderization (Geesink et al., 2006). In addition to proteolysis, connective tissue content and solubility and sarcomere length are major determinants of meat tenderness (Totland et al., 1988; Smulders et al., 1990). However, inconsistency in meat tenderness is often attributed to variations in the extent of postmortem proteolysis (Dang et al., 2020), particularly in the *longissimus* muscle (Wheeler and Koohmaraie, 1999). Hence, it is necessary to identify biomarkers related to proteolysis and tenderness in order to minimize variation in beef tenderness.

Omics approaches have been utilized in the field of meat science to examine changes in postmortem muscle and their relationship to the physicochemical and sensory properties of the resulting meat (Ramanathan et al., 2020). Proteomic profiling, in particular, has been widely exploited to gain deeper insight into the biochemical events controlling meat tenderness through providing information about protein relative abundance, modification (i.e., oxidation, degradation, and denaturation), localization, and interaction (Schilling et al., 2017). Further, proteomic studies based on comparisons between tender and tough beef have revealed several potential protein biomarkers related to tenderness (Laville et al., 2009; Bjarnadottir et al., 2012; D'Alessandro et al., 2012b; Carvalho et al., 2014). These proteins were found to be involved in different biological processes, including calcium homeostasis, oxidative stress, apoptosis, proteolysis, energy metabolism, and structural integrity. Results from these studies have shown a promising potential of proteomic analysis to improve our understanding of factors controlling meat tenderness.

Tandem mass tag (**TMT**) is a chemical labeling technique that allows for high-throughput and multiplexed quantitative proteomic analysis (O'Brien et al., 2018). TMT-based quantitative proteomics has been recently utilized in meat science research to assess differences in color stability of different ovine (Li et al., 2018) and bovine (Zhai et al., 2020) muscles. This tool has also been used to gain deeper insight into factors controlling the rate and extent of postmortem metabolism (Zequan et al., 2021; Zhu et al., 2021c). In this study, we utilized a TMT-based quantitative proteomic analysis to examine differences in protein profiles between two groups of beef steaks varying in tenderness, with the intention to identify potential biomarkers related to tenderness. Although similar studies have been previously performed (Laville et al., 2009; Bjarnadottir et al., 2012; D'Alessandro et al., 2012b; Carvalho et al., 2014), none of these studies utilized TMT-based proteomic analysis.

Materials and Methods

Muscle sampling

Market weight steers (18 ± 2 mo, n = 99) of similar genetic background, feeding, and management conditions were humanely harvested at a federally inspected commercial abattoir following USDA guidelines. At ~30 min postmortem, ~10 g of the *longissimus lumborum* (**LL**) muscle was collected from one side of each carcass, snap-frozen in liquid nitrogen, and stored at −80 °C (subsequently referred to as 0 h postmortem samples). All carcasses were stored at 4 °C for 48 h. Afterward, the rest of the LL muscle was excised from all carcasses, vacuum packaged, and aged for an additional 14 d (16 d [384 h] total aging period). At the end of the aging period, two 2.5-cm-thick steaks were obtained from each muscle; one steak was used for shear force determination, while the other one was frozen and stored in the same manner as described earlier.

Warner–Bratzler shear force

Warner–Bratzler shear force (**WBSF**) was evaluated using a WBSF (V-notch) attachment coupled to a TMS-Pro Texture Analyzer (Food Technology Co., Sterling, VA, USA) and in accordance with previously described procedures (Belk et al., 2015). Briefly, the 384 h aged steaks were cooked on an electric clamshell grill to an internal temperature of 71 °C. After cooking, steaks were blotted dry, equilibrated to room temperature, and stored overnight at 4 °C. On the following day, seven 1.27 cm-diameter core samples were collected from each steak parallel to the muscle fiber orientation with a hand-held coring device. Cores were then sheared perpendicular to the longitudinal axis of the muscle fibers. Shear force was determined as the average maximum force (Newton, N) of the seven cores. Based on WBSF measurements, 16 samples with the highest and lowest values were selected to be used for proteomic analysis in this study (n = 8 per group). These 16 samples are the same sample used in our previous publication (Dang et al., 2020).

Proteomic analysis

Protein solubilization and quantification

Frozen 0 and 384 h muscle samples were pulverized under liquid nitrogen using a mortar and pestle, and an aliquot (~100 mg) was collected from each sample in a microcentrifuge tube. Then, a buffer containing 8 M urea, 50 mM HEPES, and 1% SDS (pH 8) was added to each sample at a 1:10 ratio. The resulting mixture was homogenized with a bead-beating homogenizer (TissueLyser LT, Qiagen, Hilden, Germany) and 4 mm diameter stainless steel beads (Next Advance Inc., Troy, NY, USA). Aliquots for protein quantification were removed from the tissue homogenate, heated at 90 °C for 5 min, and centrifuged at 10,000 × g for 10 min at room temperature. Protein concentration was determined on the resulting supernatants using the RCDC protein assay kit in accordance with the manufacturer's directions (BioRad Laboratories, Hercules, CA, USA).

Protein reduction, alkylation, and digestion

All procedures were performed according to the TMT 6-plex kit manual (Thermo Fisher Scientific, Waltham, MA, USA). In brief, an aliquot from each sample corresponding to 100 µg protein was mixed with 100 mM triethyl ammonium bicarbonate (**TEAB**) to achieve a final volume of 100 µL. For reduction of disulfide bonds, Tris (2-carboxyethyl) phosphine (9.5 mM final concentration) was added and the mixture was incubated at 55 °C for 1 h. Free cysteines were then alkylated with iodoacetamide (17.9 mM final concentration) at room temperature in the dark for 30 min. Following, ice-cold

acetone was added at 6:1 ratio and proteins were precipitated overnight at −80 °C. Precipitates were collected following centrifugation at 8,000 × *g* for 10 min at 4 °C. Supernatants were removed and pellets were allowed to air dry under a sheet of foil. Pellets were reconstituted in 100 µL TEAB and then digested with 2.5 µg trypsin overnight at 37 °C.

Peptide labeling

The total peptide concentration of digested samples was determined using a NanoDrop (ND-1000; Thermo Fisher Scientific) at 205 nm (Scopes, 1974). Samples were randomly assigned to one of six TMT labels across seven TMT sets. A quality control (**QC**) sample was included in each set to account for variation between TMT sets. The QC sample consisted of all samples utilized in this study pooled into one tube at equal peptide concentrations. About 74 µg peptide from each sample was mixed with 100 mM TEAB to achieve a final volume of 100 µL. Subsequently, 41 µL of TMT label reagent was added to each sample and incubated at room temperature for 1 h. Hydroxylamine was added to each sample (0.37% final concentration) before being incubated for an additional 15 min. Samples within each TMT set were then pooled together. Peptides within each pooled sample were acidified using 15 µL of 10% trifluoracetic acid.

Peptide cleanup and fractionation

Removal of excess TMT label, desalting, and fractionation of peptides was carried out using Pierce High pH Reversed-Phase Peptide Fractionation Kit following the manufacturer's instructions (Thermo Fisher Scientific). In brief, 100 µg of the total peptide from each pooled sample was subjected to stepwise fractionation using an elution buffering (0.1% TEA and acetonitrile added from 10% to 50% final concentration) and centrifugation at 3,000 × *g* for 2 min at room temperature. A total of eight fractions were collected from each TMT set and subsequently dried using a Savant SpeedVac (Savant Instruments, Farmingdale, NY, USA). Dried samples were then reconstituted in a solution consisting of 5% acetonitrile and 0.1% formic acid.

Mass spectrometry analysis

A total of 0.75 µg of peptides was purified and concentrated from the peptide fractions using an online enrichment column (Waters Symmetry Trap C18 100 Å, 5 µm, 180 µm ID × 20 mm column). Chromatographic separation of the resulting peptide concentrates was performed using a reverse-phase nanospray column (Peptide BEH C18; 1.7 µm, 75 µm ID × 150 mm column; Waters, Milford, MA, USA) with an 85 min linear gradient from 5% to 40% (99.9% acetonitrile and 0.1% formic acid) followed by 40% to 85% for 7 min at a flow rate of 350 nL/min. Eluted samples were subjected to mass spectrometry analysis using a mass spectrometer (Orbitrap Velos Pro; Thermo Fisher Scientific) equipped with a Nanospray Flex ion source. Fragmentation was achieved via high-energy collisional dissociation with a set collision energy of 35%. Spectra were collected over an *m*/*z* range of 400 to 1,500 with only the top 10 ions possessing charge state +2 or higher accepted for MS/MS. The selection criteria were based on a dynamic exclusion limit of 1 MS/MS spectra of a given *m*/*z* value for 30 s (exclusion duration of 120 s). Fourier transform profile mode with resolutions of 30,000 and 7,500 was used for both MS and MS/MS, respectively. From the resulting spectra, a list of compounds with set S/N threshold of 1.5 and 1 scan per group was generated using the Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA).

Data processing

Tandem mass spectra extraction, charge state deconvolution, and deisotoping were processed with ProteoWizard MsConvert (Chambers et al., 2012). Spectra from all samples were searched using Mascot version 2.6.0 (Matrix Science, London, UK) against the Bos *taurus* proteome (UP000009136) and cRAP common contaminants database (75,997 total entries), specifying the digestion enzyme trypsin with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 25 ppm. Fixed modifications were set as carbamidomethylation of cysteine and TMT 6-plex of lysine and the n-terminus, while variable modifications were set as deamidation of asparagine and glutamine and oxidation of methionine.

Search results from each TMT set were subjected to MuDPIT analysis through the Scaffold software (Version 5.0; Proteome Software Inc., Portland, OR, USA; Searle et al., 2008). A peptide threshold of 89% was set so that hits to the reverse database would obtain a peptide false discovery rate (**FDR**) of 0.08% (Käll et al., 2008). Protein identifications were accepted if the protein probabilities assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003) were greater than 99.0% and contained at least two identified peptides. Proteins with similar peptides and that could not be differentiated by MS/MS analysis were grouped to satisfy the principles of parsimony. Purity correction was performed using purity values supplied by Thermo Fisher Scientific (Lots VJ313112 and VJ307214) with an algorithm described by Shadforth et al. (2005). Log-transformation and normalization of intensities were performed across samples following methods provided by Oberg et al. (2008), with transformed values being weighted by an adaptive intensity weighting algorithm. Peptide spectra that were assigned to multiple proteins and missing reference values were removed from the analysis.

Statistical analysis

Data were analyzed using Scaffold software. Pairwise comparison between tenderness categories (very tender [**VT**] vs. intermediate tender [**IT**]) and aging periods (0 vs. 384 h) was evaluated using a moderated *t*-test. A fold change threshold of 1.2 was applied to ensure confidence in differentially expressed proteins. To control for FDR and multiple testing, a Benjamini–Hochberg adjustment was performed with $P < 0.05$ (Benjamini and Hochberg, 1995).

Results

In this study, steaks of the highest and lowest WBSF values were selected for proteomic analysis ($n = 8$ per group). These samples were previously utilized by Dang et al. (2020) in a separate study that investigated metabolic differences between the two steak groups. The average WBSF value for the first group was ~49.5 N, while the second group had an average value of ~24.6 N. Destefanis et al. (2008) classified beef tenderness, by correlating WBSF values and sensory scores, into five categories: very tough (>63 N), tough (53 to 63 N), intermediate (43 to 53 N), tender (33 to 43 N), and VT (<33 N). Following this classification, the first group of steaks was categorized as IT, while the second group was VT.

From the TMT-based proteomic analysis, a total of 876 proteins were identified, of which 51 proteins were differentially abundant ($P < 0.05$) across tenderness categories and aging periods (IT at 0 h vs. VT at 0 h; IT at 0 h vs. IT at 384 h; VT at 0 h vs. VT at 384 h; IT at 384 h vs. VT at 384 h). To simplify the results, differentially abundant proteins were categorized based on their main biological processes into eight different categories: proteins involved in muscle contraction and calcium signaling (Table 1), carbohydrate metabolism, TCA cycle and oxidative phosphorylation, and fatty acid metabolism (Table 2), extracellular matrix organization and platelet activation (Table 3), and a category for other biological functions (Table 4).

Proteins involved in muscle contraction and calcium signaling

Fourteen proteins involved in muscle contraction were differentially abundant between the tenderness categories and aging periods (Table 1). Myosin light chain 6B (**MYL6B**) and alpha-actin (**ACTA1**) were more abundant ($P = 0.008$) in the VT than IT steaks at 0 h postmortem. Abundance of myosin light chain 3 (**MYL3**) increased ($P = 0.0004$) in the VT steaks after 384 h of aging, whereas myosin regulatory light chain 2 (**MYL2**), synaptopodin 2 like (**SYNPO2L**), telethonin (**TCAP**), myozenin 3 (**MYOZ3**), vimentin (**VIM**), and PDZ and LIM domain protein 7 (**PDLIM7**) were all found to be less abundant ($P \leq 0.008$) in the VT steaks at 384 h compared with their 0 h counterparts. Similarly, a decrease ($P \leq 0.01$) in the abundance of TCAP, MYOZ3, VIM, and PDLIM7 was observed in the IT steaks following aging. On the other hand, aging of the IT steaks increased ($P \leq 0.001$) the abundance of myosin heavy chain I (which is encoded by MYH7), MYL6B, four and a half LIM domains 1 (**FHL1**), cysteine and glycine-rich protein 3 (**CSRP3**), and calsequestrin (**CASQ2**). At 384 h postmortem, transgelin (**TAGLN**) and CSRP3 were more abundant ($P = 0.001$) in the IT steaks than VT.

Among the differentially identified proteins, four were related to calcium signaling (Table 1). Annexin A2 (**ANXA2**) was greater ($P = 0.0005$) in the IT steaks than the VT at 0 h postmortem. Decreased abundance ($P = 0.04$) of protein S100-A4 (S100A4) was detected in the VT steaks after 384 h of aging. Similarly, aging lowered the abundance ($P \leq 0.0004$) of cadherin-13 (**CDH13**), ANXA2, and protein S100-A2 (**S100A2**) in the IT steaks.

Proteins related to metabolism in skeletal muscle

Lactate dehydrogenase B (**LDHB**), mannose-6-phosphate isomerase (**MPI**), 6-phosphogluconate dehydrogenase (**PGD**), transketolase (**TKT**), and NADH-cytochrome b5 reductase 3 (**CYB5R3**) are proteins involved in carbohydrate metabolism

Table 1. Differentially abundant proteins involved in muscle contraction and calcium signaling between IT and VT beef steaks at 0 and 384 h postmortem[1]

Biological process	Gene name	Log_2 fold change (IT vs. VT) at 0 h	Log_2 fold change (VT 384 h vs. VT 0 h)	Log_2 fold change (IT 384 h vs. IT 0 h)	Log_2 fold change (IT vs. VT) at 384 h
Muscle contraction					
Transgelin	TAGLN				0.27**
Myosin heavy chain I	MYH7			0.28***	
Myosin regulatory light chain 2	MYL2		−0.58*		
Myosin light chain 3	MYL3		0.29***		
Myosin light chain 6B	MYL6B	−0.28***		0.51***	
Actin, alpha skeletal muscle	ACTA1	−0.47**			
Synaptopodin 2 like	SYNPO2L		−0.28***		
Telethonin	TCAP		−0.44**	−0.37*	
Myozenin 3	MYOZ3		−0.42***	−0.37***	
Vimentin	VIM		−0.29***	−0.45***	
PDZ and LIM domain protein 7	PDLIM7		−0.28***	−0.33***	
Four and a half LIM domains 1	FHL1			0.29***	
Cysteine and glycine-rich protein 3	CSRP3			0.3***	0.41**
Calsequestrin	CASQ2			0.27***	
Calcium signaling					
Cadherin-13	CDH13			−0.35***	
Annexin A2	ANXA2	0.28***		−0.42***	
Protein S100-A2	S100A2			−0.63***	
Protein S100-A4	S100A4		−0.3*		

[1]Data are presented as Log_2 fold change.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

Table 2. Differentially abundant proteins involved in carbohydrate, aerobic, and fatty acid metabolism between IT and VT beef steaks at 0 and 384 h postmortem[1]

Biological process	Gene name	Log_2 fold change (IT vs. VT) at 0 h	Log_2 fold change (VT 384 h vs. VT 0 h)	Log_2 fold change (IT 384 h vs. IT 0 h)	Log_2 fold change (IT vs. VT) at 384 h
Carbohydrate metabolism					
L-lactate dehydrogenase B chain	LDHB	−0.3***			
Mannose-6-phosphate isomerase	MPI	−0.27*			
6-phosphogluconate dehydrogenase, decarboxylating	PGD			−0.43*	
Transketolase	TKT		−0.38***		
NADH-cytochrome b5 reductase 3	CYB5R3			−0.31***	
TCA cycle and oxidative phosphorylation					
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial	NDUFB11			0.28**	
Cytochrome c oxidase subunit 5A, mitochondrial	COX5A		0.28***		
Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	IDH3B				−0.3**
Fatty acid metabolism					
Fatty acid synthase	FASN			−0.44**	
Fatty acid-binding protein, heart	FABP3			0.29**	
Fatty acid-binding protein, adipocyte	FABP4		−0.33*		
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADM			0.31***	
Carbonyl reductase family member 4	CBR4			0.28*	
Perilipin 4	PLIN4		−0.45***	−0.4***	

[1]Data are presented as Log_2 fold change.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

and were identified as being differentially abundant in this study (Table 2). LDHB and MPI were expressed at a greater level ($P \leq 0.01$) in the VT category compared with IT at 0 h. Lower abundance ($P = 0.0004$) of TKT was observed in the VT steaks following aging, while IT stakes had decreased abundance ($P \leq 0.03$) of PGD and CYB5R3.

Three mitochondrial proteins involved in aerobic metabolism, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11 (**NDUFB11**), cytochrome c oxidase subunit 5A (**COX5A**), and isocitrate dehydrogenase [NAD] subunit beta (**IDH3B**), were differentially expressed (Table 2). At 384 h postmortem, VT steaks had greater COX5A abundance ($P = 0.0004$) in comparison to their 0 h counterparts, and the same effect ($P = 0.001$) was observed for NDUFB11 in the IT samples. When the two steak categories were compared at 384 h postmortem, IDH3B was greater ($P = 0.001$) in the VT samples.

Six proteins involved in fatty acid metabolism were identified to be differentially abundant between the two aging periods (Table 2). Fatty acid-binding protein 4 (**FABP4**) and perilipin 4 (**PLIN4**) were more abundant ($P \leq 0.02$) in the VT steaks at 0 h compared with their 384 h aged counterparts. Similarly, more abundance ($P \leq 0.003$) of fatty acid synthase (**FASN**) and PLIN4 was observed at 0 h in the IT steaks. Conversely, lower abundance ($P \leq 0.01$) of fatty acid-binding protein 3 (**FABP3**), medium-chain specific acyl-CoA dehydrogenase (**ACADM**), and carbonyl reductase family member 4 (**CBR4**) was detected in the IT samples after aging.

Extracellular matrix and platelet activation proteins

IT steaks at 0 h postmortem possessed greater expression ($P = 0.01$) of two extracellular matrix proteins, collagen alpha-1(I) chain (**COL1A1**) and collagen alpha-2(I) chain (**COL1A2**), than VT steaks (Table 3). Aging for 384 h lowered the abundance ($P \leq 0.04$) of the extracellular matrix proteins COL1A1, COL1A2, and collagen alpha-1 (III; **COL3A1**), lumican (LUM), and prolargin (PRELP) in the VT steaks. A similar effect was also observed in the IT steaks, in which abundance of COL1A1, COL1A2, COL3A1, decorin (**DCN**), fibromodulin (**FMOD**), and PRELP decreased ($P \leq 0.02$) following aging.

Platelet activation proteins, fibrinogen alpha chain (**FGA**), fibrinogen beta chain (**FGB**), and fibrinogen gamma-B chain (**FGG**), were all greater ($P \leq 0.008$) in the IT steaks compared with VT at 0 h postmortem (Table 3). A decrease in the abundance ($P \leq 0.0004$) of FGB and FGG in the IT steaks was observed after 384 h of aging.

Proteins involved in other biological functions

Proteins involved in seven other biological functions were also found differentially abundant in the present study (Table 4). The abundance of two adrenergic signaling proteins, protein phosphatase 1 regulatory inhibitor subunit 1A (**PPP1R1A**) and protein phosphatase inhibitor 2 (**PPP1R2**), were observed to be decreased ($P \leq 0.004$) following aging in both steak categories. At 0 h postmortem, VT steaks possessed a greater level ($P = 0.0007$) of transferrin receptor

Table 3. Differentially abundant proteins involved in an extracellular matrix organization and platelet activation between IT and VT beef steaks at 0 and 384 h postmortem[1]

Biological process	Gene name	Log_2 fold change (IT vs. VT) at 0 h	Log_2 fold change (VT 384 h vs. VT 0 h)	Log_2 fold change (IT 384 h vs. IT 0 h)	Log_2 fold change (IT vs. VT) at 384 h
Extracellular matrix organization					
Collagen alpha-1(I) chain	COL1A1	0.27*	−0.42***	−0.51***	
Collagen alpha-2(I) chain	COL1A2	0.35*	−0.29***	−0.55***	
Collagen alpha-1(III) chain	COL3A1		−0.49*	−0.45**	
Decorin	DCN			−0.32***	
Fibromodulin	FMOD			−0.46*	
Lumican	LUM		−0.32**		
Prolargin	PRELP		−0.27***	−0.3***	
Platelet activation					
Fibrinogen alpha chain	FGA	0.31***			
Fibrinogen beta chain	FGB	0.33***		−0.27***	
Fibrinogen gamma-B chain	FGG	0.37**		−0.57***	

[1]Data are presented as Log_2 fold change.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

protein 1 (**TFRC**; a mineral transport protein) than IT steaks. A decrease in the abundance ($P = 0.04$) of copper transport protein (**ATOX1**) in the VT steaks was observed after aging. Greater expression ($P = 0.0005$) of carbonic anhydrase 3 (**CA3**), a protein involved in acid-base balance, was detected at 0 h postmortem in the VT steaks compared with the IT steaks. At 384 h postmortem, a chaperonin protein, heat shock protein beta-7 (**HSPB7**), was more abundant ($P = 0.006$) in the IT steaks than their VT counterparts. Aging for 384 h lowered the abundance ($P \leq 0.02$) of the cochaperonin protein alanyl-tRNA editing protein Aarsd1 (**AARSD1**) and the ubiquitination protein NEDD8-conjugating enzyme Ubc12 (**UBE2M**) in the VT and IT steaks, respectively. Two apoptosis-related proteins were found to be differentially expressed in this study. Greater expression ($P = 0.007$) of glutathione S-transferase mu 1 (**GSTM4**) was observed in the VT steaks than IT steaks at 0 h postmortem. At 384 h postmortem, an increase in the abundance ($P = 0.001$) of glioblastoma amplified sequence (**GBAS**) was detected in the VT steaks.

Discussion

Proteins involved in muscle contraction and calcium signaling

In the present study, a greater abundance of MYL6B and ACTA1 was detected in the VT steaks at 0 h postmortem compared with their IT counterparts (Table 1). However, this difference was abolished after aging, suggesting greater degradation of these two proteins in the VT steaks. MYL6B plays structural and functional roles within the sarcomere by supporting the neck region of myosin and modulating the interaction between myosin head and actin, respectively (Franco et al., 2015). Degradation of MYL6B and ACTA1 compromise stability of the actomyosin cross-bridges (Huang et al., 2020), thereby leading to the loss of meat structural integrity and enhancement of tenderness (Wang et al., 2013; Malheiros et al., 2019). Malheiros et al. (2019) reported increased oxidative damage of MYL6B and ACTA1 during aging in tender beef steaks compared with tough steaks. These authors suggested that increased susceptibility for oxidative damage may improve the potential for postmortem proteolysis, which may explain the greater degradation of MYL6B and ACTA1 in the VT steaks. Using the same samples used in the current study, we previously observed that the VT steaks encountered greater proteolysis following aging than the IT steaks (Dang et al., 2020).

Aging for 384 h lowered the abundance of several structural and contractile proteins in both the IT and VT steaks (Table 1). This includes the intermediate filament protein VIM and the structural assembly-related proteins TCAP, MYOZ3, and PDLIM7. The reduction in the abundance of these proteins after aging is likely due to postmortem proteolysis. VIM is an intermediate filament that exists at the periphery of the Z-line and closely interacts with cytoskeletal proteins (Baykal et al., 2018). TCAP is a sarcomeric protein that plays a crucial role in muscle assembly by anchoring titin to the Z-line (Lee et al., 2006). Both VIM and TCAP are substrates for calpain-1 (Nelson and Traub, 1983; Lian et al., 2013) and, as such, their postmortem proteolysis may contribute to meat tenderization. Similarly, MYOZ3 and PDLIM7 are also sarcomeric proteins that are associated with Z-line assembly (Kim et al., 2019; Zhu et al., 2021a). Boudon et al. (2020) observed a negative association between the expression of MYOZ3 and tenderness of bovine *longissimus* steaks. Others have also found

Table 4. Differentially abundant proteins associated with several other biological functions between IT and VT beef steaks at 0 and 384 h postmortem[1]

Biological process	Gene name	Log_2 fold change (IT vs. VT) at 0 h	Log_2 fold change (VT 384 h vs. VT 0 h)	Log_2 fold change (IT 384 h vs. IT 0 h)	Log_2 fold change (IT vs. VT) at 384 h
Adrenergic signaling					
Protein phosphatase 1 regulatory inhibitor subunit 1A	PPP1R1A		−0.44***	−0.47***	
Protein phosphatase inhibitor 2	PPP1R2		−0.9**	−0.55**	
Mineral transport					
Copper transport protein ATOX1	ATOX1		−0.28*		
Transferrin receptor protein 1	TFRC	−0.32***			
Acid-base balance					
Carbonic anhydrase 3	CA3	−0.27***			
Chaperonin					
Heat shock protein beta-7	HSPB7				0.35**
Cochaperonin					
Alanyl-tRNA editing protein Aarsd1	AARSD1		−0.42*		
Ubiquitination					
NEDD8-conjugating enzyme Ubc12	UBE2M			−0.52*	
Apoptosis-related factors					
Glioblastoma amplified sequence	GBAS		0.29**		
Glutathione S-transferase mu 1	GSTM4	−0.32**			

[1]Data are presented as Log_2 fold change
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

a similar association between meat tenderness and the abundance of PDLIM7 (Gagaoua et al., 2020a; Zhu et al., 2021a). Therefore, postmortem degradation of MYOZ3 and PDLIM7 may contribute to meat tenderization.

On the other hand, MYH7, MYL6B, FHL1, CSRP3, and CASQ2 were more abundant in the IT steaks at 384 h than 0 h postmortem. A decrease in total muscle proteome is usually observed during the postmortem aging of meat (Della Malva et al., 2022). Because protein abundance is expressed relative to the total proteome when using a gel-free proteomic approach, the increase in the abundance of the aforementioned proteins is likely due to a decrease in total proteome variance in the IT samples. On the other hand, a decrease in protein relative abundance indicates a faster rate of degradation relative to the total proteome. Although degradation of myosin is not thought to occur in postmortem muscle (Bandman and Zdanis, 1988; Koohmaraie, 1994), several studies within the last decade have indicated that myosin is prone to degradation during aging (Laville et al., 2009; Anderson et al., 2012; Fu et al., 2020). It was proposed by Fu et al. (2020) that myosin oxidation by reactive oxygen species (**ROS**) during meat aging enhances its susceptibility to proteolysis. Myosin heavy chain 1 (**MyHC-1**; encoded by the MYH7 gene) is a contractile protein that is found in type I muscle fibers (slow-twitch oxidative fibers). Guillemin et al. (2011) observed a negative relationship between the abundance of MyHC-I and tenderness of beef *longissimus* steaks at 14 d postmortem. Similarly, from our previous study that utilized the same steaks used in this study, a greater abundance of MyHC-I was observed in the IT steaks (Dang et al., 2020). FHL1 is localized at the Z-line of myofibers, and its degradation during aging weakens the Z-line and enhances meat tenderness (Picard et al., 2019). A greater abundance of this protein in the IT steaks following aging suggests better intactness of muscle contractile elements. Indeed, a positive association between FHL1 and beef toughness following aging has been previously reported (Laville et al., 2009). CSRP3, also known as muscle LIM protein, primarily assists in the development of muscle architecture during animal growth and development (Rashid et al., 2015), and has been used as a biomarker of meat toughness (Bernard et al., 2007; Zapata et al., 2009; Boudon et al., 2020). CASQ2 is a calcium-binding protein that is part of the sarcoplasmic reticulum calcium-regulatory proteins (Purslow et al., 2021). There are two isoforms of CASQ2 in skeletal muscle, calsequestrin-1 and -2. Notably, CASQ2 has been shown to be more abundant in slow-oxidative muscle fibers (Beard et al., 2004; D'Adamo et al., 2016). In addition, it was postulated that greater expression of CASQ2 decreases cytosolic calcium levels in postmortem muscle; hence, reducing the activity of calpain-1 and ultimately the extent of postmortem proteolysis (Marrocco et al., 2011). Overall, these data suggest greater intactness of muscle contractile elements in the IT steaks (Morzel et al., 2004), which likely contributed to the differences in tenderness between the two steaks categories.

A greater abundance of CSRP3 and TAGLN was seen in the IT steaks compared with their VT counterparts at 384 h postmortem (Table 1). In agreement with this, Zapata et al. (2009) found a positive association between the abundance of CSRP3 and WBSF values in 14 d aged beef steaks. TAGLN is an actin-binding protein that is a member of the calponin family of proteins (Lawson et al., 1997). TAGLN increases filament rigidity and protects against the weakening of the cytoskeletal structure (Assinder et al., 2009). Hence, a greater abundance of TAGLN in aged meat may indicate limited proteolysis. Yet, to the best of my knowledge, no previous studies have evaluated the relationship between the abundance of TAGLN and the tenderness of meat.

At 0 h postmortem, IT steaks had greater expression of ANXA2 than VT steaks (Table 1). Annexins are a family of proteins that are expressed in all eukaryotic cells and participate in a variety of cellular processes (Gerke and Moss, 2002). It has been proposed that annexins play a role in buffering the intracellular calcium and possess anti-apoptotic capabilities (Ouali et al., 2013; Longo et al., 2015), which may potentially hinder the activation of the calpain and caspase proteolytic systems (Picard and Gagaoua, 2017). Hence, an increased relative abundance of ANXA2 in the IT steaks may have contributed to the limited degradation of myofibrillar proteins in this category compared with the total proteome (Table 1).

A decrease in S100A4 abundance was observed in the VT steaks during aging (Table 1), indicating a faster rate of degradation relative to the total proteome. Similarly, the abundance of CDH13, ANXA2, and S100A2 decreased after 384 h of aging in the IT steaks. Protein S100A2 and S100A4 are calcium-binding proteins that are involved in the regulation of muscle contraction and organization of the cytoskeleton (Barraclough, 1998; Zhai et al., 2020). CDH13 is responsible for selective cell recognition and adhesion in skeletal muscle (Redfield et al., 1997; Roman-Gomez et al., 2003). To our knowledge, no previous reports have associated the abundance of S100A2, S100A4, and CDH13 with meat tenderness. Thus, additional work is warranted if we are to confidently exploit their involvement in the development of meat tenderness during aging.

Proteins related to predominant metabolism in skeletal muscle

Skeletal muscles are comprised of different proportions of functionally diverse muscle fibers that are classified based on contractile force/speed and predominant metabolism (Brooke and Kaiser, 1970; Schiaffino et al., 1989). Previous studies revealed three different fiber types in adult bovine skeletal muscle: type I, IIA, and IIX (Kim et al., 2017). Type I fibers are classified as having greater oxidative capacity compared with their glycolytic counterparts, Type IIX fibers (McGilchrist et al., 2016). Type IIA fibers are oxidative-glycolytic and possess intermediate properties between type I and IIX (Joo et al., 2017).

MPI and LDHB are two enzymes involved in carbohydrate metabolism and intimately associated with glycolysis. These enzymes were found to be more abundant in the VT steaks at 0 h postmortem in comparison to the IT steak (Table 2), whereas differences in oxidative enzymes were not detected between the two categories. MPI is responsible for the conversion of mannose 6-phosphate to fructose 6-phosphate, which is an intermediate metabolite for glycolysis. LDH subunit B is one of the two subunits that make up the tetrameric structure of LDH. This glycolytic enzyme catalyzes the reduction of pyruvate into lactate (Melkonian and Schury, 2019). Various conclusions have been made regarding the use of glycolytic enzymes as biomarkers of meat tenderness. Several proteomic studies have suggested that greater expression of glycolytic proteins is positively associated with beef tenderness (Silva et al., 2019; López-Pedrouso et al., 2021), while others have shown a negative correlation (Zapata et al., 2009; Picard et al., 2018). In general, however, greater expression of proteins related to glycolysis is associated with more tender meat (Gagaoua et al., 2020a). Glycolytic muscle fibers exhibit a faster rate of ATP depletion in comparison to oxidative fibers (Matarneh et al., 2017). The sarcoplasmic reticulum calcium pump, the enzyme responsible for sequestering cytosolic calcium into the sarcoplasmic reticulum, requires ATP to function. Hence, a faster rate of ATP depletion would, in turn, accelerate the increase in cytosolic calcium levels and thereby calpain-1 activation (Rhee and Kim, 2001). Additionally, calpastatin, the endogenous inhibitor of calpain-1, is generally less abundant in glycolytic muscle (Ouali and Talmant, 1990; Choi and Kim, 2009), which increases calpain-1 proteolytic activity. These results correspond to what we previously reported (Dang et al., 2020), in which VT steaks had greater glycolytic potential and postmortem proteolysis than the IT steaks.

The abundance of several proteins (TKT, FABP4, and PLIN4) involved with carbohydrate and fatty acid metabolism decreased due to aging in the VT steaks, while COX5A increased (Table 2). In the IT steaks, a lower abundance of PGD, CYB5R3, FASN, and PLIN4 was observed after aging. On the other hand, a greater abundance of NDUFB11, FABP3, ACADM, and CBR4 was detected following aging. As described earlier, the increase in the abundance of proteins involved in energy metabolism during aging is likely due to the decrease in total muscle proteome, while the decrease in the abundance is a function of postmortem proteolysis. Nonetheless, the breakdown of these non-myofibrillar proteins may not contribute to postmortem meat tenderization, as tenderization is typically attributed to the degradation of myofibrillar proteins. Regardless, these proteins might be viewed as indicators of the extent of postmortem proteolysis.

Extracellular matrix and platelet activation proteins

COL1A1 and COL1A2 are involved with extracellular matrix organization; together they assemble to form the triple helix structure of type I collagen (Henriksen and Karsdal, 2016; Liu et al., 2016). Increased expression of these two proteins in the IT steaks at 0 h postmortem (Table 3) suggests that the difference in tenderness between the two categories is, in part, due to a greater proportion of connective tissue content. Using a proteomic approach, Bjarnadottir et al. (2012) observed a negative association between COL1A1 and COL1A2 and beef tenderness, which corresponds to the results of the current study. The decrease in the abundance of several extracellular matrix proteins in both tenderness categories following aging (Table 3) is probably due to the presence of metalloproteinases in the extracellular matrix. Metalloproteinases are a family of zinc-dependent endopeptidases that are involved in skeletal muscle tissue remodeling and repair (Nagase and Woessner, 1999). While some studies have suggested the involvement of metalloproteinases in postmortem tenderization (Sylvestre et

al., 2002; Purslow et al., 2012), their exact role has not been thoroughly explored.

Fibrinogen complex proteins (FGA, FGB, and FGG) were expressed at a greater level in the IT than the VT steaks at 0 h postmortem (Table 3). A reduction in the abundance of FGB and FGG was observed in the IT samples following aging in comparison to their nonaged counterparts. Fibrinogen is a vascular extracellular matrix protein that functions as a clotting and adhesive agent (Mosesson, 2005). While the influence of fibrinogen on beef tenderness is poorly understood, bovine fibrinogen has three high-affinity and several low-affinity calcium-binding sites (Marguerie et al., 1977), which may limit the increase in cytosolic calcium concentration and subsequently calpain-1 activity. Gagaoua et al. (2020b) indicted that fibrinogen complex proteins are degraded during the postmortem period, and might be used as indicators of the extent of postmortem proteolysis.

Proteins involved in other biological functions

There are two apoptotic pathways through which apoptosis is initiated: the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. While these two signaling pathways are interconnected (Igney and Krammer, 2002), the mitochondrial apoptotic pathway has been regarded as the main apoptotic pathway involved in the conversion of muscle to meat (Huang et al., 2016). Unfavorable conditions in postmortem muscle trigger mitochondrial dysfunction (Arnoult et al., 2005), which eventually leads to the activation of the apoptotic caspases (Denecker et al., 2000). While activation of the caspase system, specifically caspase-3, has been associated with improvement in meat tenderness (Chen et al., 2015; Dang et al., 2022), a greater abundance of antiapoptotic proteins such as heat shock proteins (**HSP**) is usually associated with less tender meat (Balan et al., 2014; Lomiwes et al., 2014; Ma and Kim, 2020).

GSTM4 is part of a family of detoxification enzymes involved in the quenching of ROS in skeletal muscle (Samanta et al., 2014). A greater expression of GSTM4 was detected in the VT steaks than IT steaks at 0 h postmortem (Table 4). Increased expression of GSTM4 is an indication of the inability of a muscle cell to maintain reducing conditions (i.e., increased levels of ROS; Poleti et al., 2018). On the other hand, excess ROS in the cellular system has been associated with greater activity of the apoptotic protease caspase-3 and improved beef tenderness (Dang et al., 2022). Zhu et al. (2021b) indicated that tenderness and juiciness of beef steaks are positively correlated with the expression of GSTM4. Thus, apoptotic-mediated proteolysis could be another contributor to the increased tenderness of the VT steaks.

Due to their antiapoptotic properties, HSPs have been proposed as potential determinants of the extent of postmortem proteolysis (Carvalho et al., 2014; Lomiwes et al., 2014; Thornton et al., 2017; Briggs et al., 2021). HSPs operate as molecular chaperones in muscle cells and play a role in stress resistance (Ozawa et al., 2000). Under postmortem conditions, the state of anoxia and oxidative stress trigger HSPs to mitigate the degradation of myofibrillar proteins by endogenous proteases (Lomiwes et al., 2014). In the present study, HSPB7, a member of the small HSP (sHSP) family, was more abundant in the IT steaks than VT steaks at 384 h postmortem (Table 4). It has been suggested that HSPB7 role in muscle is to maintain myofiber structure and integrity during periods of oxidative stress. Furthermore, several studies have shown a negative relationship between sHSP and meat tenderness in beef (Kim et al., 2008; D'Alessandro et al., 2012a; Picard et al., 2014).

CA3 is an enzyme that catalyzes the reversible conversion of carbon dioxide to bicarbonate in the muscle (Malheiros et al., 2019), and has been utilized as a predictor of meat tenderness (Schilling et al., 2017). In this study, a greater expression of CA3 was observed in the VT steaks than the IT steaks at 0 h postmortem (Table 4). Similarly, D'Alessandro et al. (2012a) reported greater expression of CA3 in more tender beef steaks compared with tougher steaks.

A greater expression of TFRC was detected in the VT steaks at 0 h postmortem. A decrease in the abundance of adrenergic signaling (PPP1R1A and PPP1R2), mineral transport (ATOX1), cochaperonin (AARSD1), and apoptosis (GBAS)-related proteins was observed in the VT steaks following aging. Similarly, a decrease in abundance of PPP1R1A, PPP1R2, and UBE2M occurred in the IT steaks after aging. However, the involvements of these proteins in the development of meat tenderness need further investigation.

Conclusions

The findings presented in this study indicate differences in protein profiles between the VT and IT steaks. In general, a lower abundance of muscle contractile proteins was observed in the VT steaks compared with the IT steaks after aging, postulating that more proteolysis occurred in the VT steaks. In conjunction with this, a greater abundance of chaperonin and calcium-binding proteins was observed in the IT steaks which, in turn, could have limited proteolysis by calpains and other endogenous proteases. Furthermore, a greater abundance of connective tissue proteins was observed in the IT steaks, which is important to note considering that connective tissue content and solubility are associated with lower meat tenderness. Collectively, these results improve our understanding of the factors controlling meat tenderness and tenderness development during aging. Further investigation is warranted to gain insight into potential protein biomarkers related to improving meat tenderness.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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