EuPA OPEN PROTEOMICS 6 (2015) 28–41

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In-depth characterisation of the lamb meat proteome from *longissimus lumborum*

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ARTICLE INFO

Article history:
Received 19 May 2014
Received in revised form
24 December 2014
Accepted 9 January 2015
Available online 19 January 2015

Keywords:
Sheep
Lamb
Muscle
Meat
Proteomics

ABSTRACT

Lamb is one of the major red meats consumed globally, both as a key component in the diet of some countries, and as a niche meat product in others. Despite this relatively wide consumption, an in-depth description of the global protein composition of lamb has not been reported. In this study, we investigated the proteome of the 48 h post-mortem lamb *longissimus lumborum* through separation of the samples into sarcoplasmic, myofibrillar and insoluble fractions, followed by an in-depth shotgun proteomic evaluation and bioinformatic analysis. As a result, 388 ovine-specific proteins were identified and characterised. The 207 proteins found in the sarcoplasmic fraction were dominated by glycolytic enzymes and mitochondrial proteins. This fraction also contained several sarcomeric proteins, e.g., myosin light chains and titin. Some of them might be the degradation products from the post-mortem proteolysis. Actin, myosin and tropomyosin were abundant in the myofibrillar fraction while nebulin and titin were also present. Collagen type I, III and IV were found in the insoluble fraction but there were also sequences from myosin and titin. The present study also confirms the existence of at least 300 predicted protein sequences obtained from the latest issue of the sheep genome (version 3) with high confidence.

1. Introduction

Protein is a key component of meat and a critical determinant of its structure, nutritional value [1] and texture [2]. Moreover, specific proteins are involved in the post-mortem processes responsible for quality aspects of meat such as tenderness [3] and colour [4,5].

In recent years, studying meat or skeletal muscle proteins from a global view, i.e., by proteomic approaches, has become popular in the domain of meat science [6,7], especially for cattle and pigs. Proteomic information can help elucidate the complex molecular events that underlie skeletal muscle growth [8], muscle-to-meat conversion [9–13], meat quality variation [10,12,14] and the effects of meat processing [7,15,16].

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http://dx.doi.org/10.1016/j.euprot.2015.01.001

Sheep meat is widely consumed around the world [17] even though it is currently considered by some as a niche product in developed countries [18]. To New Zealand, the economic significance of sheep meat is apparent: in the year ended June 2012, sheep meat alone made up almost half of the meat export revenue, equivalent to 5.9% of the country's total export revenue [19]. Notably, lamb meat takes up the majority of New Zealand's sheepmeat export value, being approximately $1.5 billion Euros in year ended September 2012 [19]. Despite being a major global meat source, only a few proteomic papers on sheep skeletal muscle have been published [20–26] in contrast to other popular meat animal species [see the comprehensive review by 10,27]. To the best of our knowledge, no in-depth study dedicated to describing the skeletal muscle/meat proteome of lambs has been reported to date.

The final quality of meat is the result of variation in the genetics and environmental conditioning of the animal it is derived from and post-mortem changes in the days following slaughter. Among the most important of these post-mortem changes are the decline in pH and the extensive proteolysis of cytoskeletal proteins. To help deliver better utilisation of meats beyond their traditional use, the knowledge about the overall protein composition of skeletal muscle/meat is fundamental. Information from the skeletal muscle/meat proteome can also serve as reference for proteomic studies on quality variation of raw meat or protein modifications induced by different processing conditions or techniques. Furthermore, during the process of proteome identification, valuable insight can be gained for method development of hypothesis-driven expression or post-translational modification studies. For example, a 2-D gel protein map of the exudates of 1-day post-mortem porcine *longissimus thoracis et lumborum* muscles with 89 protein spots identified was established and used as a basis to discover differentially expressed proteins between phenotypes varying in water-holding capacity [28]. In addition, for organisms whose genome sequences have not yet been fully annotated, large-scale protein characterisation may provide experimental evidence for the existence of some proteins currently predicted from gene models, e.g., Santos et al. [29].

Protein maps or catalogues of the skeletal muscle have been generated for several vertebrate species such as human [30,31], cattle [32–34], pigs [35,36], rabbits [37,38], mice [39–42] and cod [43]. In this study, we aimed to characterise the proteome of lamb meat derived from *longissimus lumborum muscle*. The meat was sampled at 48 h after slaughter, when most of the physical and biochemical post-mortem changes had occurred, and then separated into sarcoplasmic, myofibrillar and insoluble fractions. As a result, 388 lamb meat protein identifications were obtained and catalogued.

2. Materials and methods

Ammonium bicarbonate, bromophenol blue, dithiothreitol (DTT), glycerol, glass beads (anti-bumping granules) and tris(hydroxymethyl)aminomethane (Tris base) were from BDH (Poole Dorset, UK). Ethanol, methanol and sodium dodecyl sulphate (SDS) were from Fisher Scientific (Hampton, NH, USA). Acrylamide and Coomassie Brilliant Blue (CBB) G-250 were from Bio-Rad (Hercules, CA, USA). Ortho-phosphoric acid (85%), acetic acid and glycine were from Merck (Darmstadt, Germany). Milli-Q water used for protein extraction and electrophoresis-related experiment was from Merck Millipore (Billerica, MA, USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and iodoacetamide were from Sigma (St. Louis, MO, USA). Sequencing grade modified porcine trypsin was from Promega (Madison, WI, USA). Acetone was from Acros Organics (Geel, Belgium). Formic acid was from Ajax Finechem (Taren Point, NSW, Australia) and Fluka (Buchs, Switzerland). LC–MS grade acetonitrile (ACN) and water were from both Fisher Scientific (Hampton, NH, USA) and Fluka (Buchs, Switzerland). The Fluka reagents mentioned above were used for LC–MS/MS gradient solvents preparation. All the organic solvents used were of HPLC grade unless otherwise indicated. The water and ACN used for in-gel digestion and MS analysis were of LC–MS grade. Water was used as solvent unless otherwise indicated.

2.1. Animals

In January 2011, pasture-fed Coopworth lambs ($n = 5$) from the Lincoln University (Canterbury, New Zealand) flock were slaughtered at approximately 12 weeks of age with the standard captive bolt stunning procedure followed by exsanguination. The carcasses were hung at room temperature for 1 h (to allow the muscles to relax) and then stored in the chiller at 8–10 °C until sampling. Samples were taken at 48 h post-mortem from the *longissimus lumborum* muscle and kept at −20 °C until analysis.

2.2. Sarcoplasmic protein extraction

Lamb meat samples of approximately equal weights from five individuals were pooled (around 0.5 g in total) and ground under liquid nitrogen. Sarcoplasmic protein was extracted from the meat powder in 40 mM Tris–Cl (pH 7.6) containing a protease inhibitor cocktail (Complete™ from Roche Diagnostics GmbH, Mannheim, Germany; one tablet/9 mL of the Tris–Cl buffer), at a meat-to-buffer ratio of 1:8 (w/v), using a hand-held, drill-type homogeniser over ice for 1 min. Homogenate was centrifuged (17,810 × *g*; 20 min; 4 °C). The supernatant, referred to as the "sarcoplasmic fraction", was stored at −80 °C until analysis. The protein concentration of the fraction was estimated using a 2D-Quant kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.3. Myofibrillar protein extraction

Around 0.4 g of the pooled meat samples was pulverised and homogenised as described in Section 2.2 but using a 1:16 meat-to-Tris buffer ratio (w/v). Homogenate (1.2 mL) aliquots were centrifuged as previously described. After removing the supernatant, the remaining crude pellet was extracted by shaking vigorously on a reciprocal shaker at room temperature with glass beads in 1.8 mL of SDS-PAGE sample buffer (62.5 mM Tris–Cl, 25% glycerol, 5% SDS, 0.0015% bromophenol blue, pH 6.8 and freshly added 350 mM DTT) for 16 h and then stored at

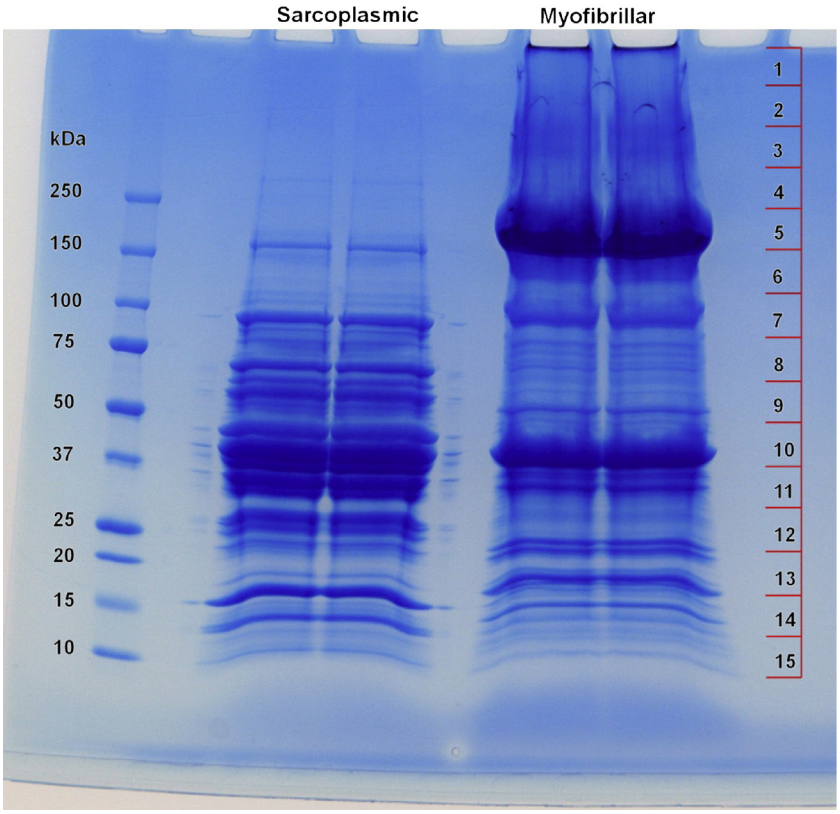

Fig. 1 – SDS-PAGE separation of lamb meat protein extracts on a 4–20% T gel. The marks on the right hand side of the image indicate the approximate position of the gel lanes sliced for proteomic analysis.

−80 °C until use. To obtain the homogenate pellet, the mixture was centrifuged at 16,000 × *g* for 25 min at 15 °C. This supernatant was referred to as the "myofibrillar fraction." The protein concentrations of each myofibrillar fraction were estimated using a 2D-Quant kit.

2.4. *SDS-PAGE*

The sarcoplasmic fraction was mixed with the SDS sample buffer at a ratio of 1:1 (v/v) and heated for 5 min at 95 °C with mild shaking. The myofibrillar fraction was heated directly in the same way. Protein fractions were separated on two 4–20% T Criterion Tris–HCl precast gels (Bio-Rad) at a constant voltage of 200 V, 80 mA and 15 W until the bromophenol blue dye front was about to reach the bottom of the gel. For Gel 1, 90 μg of sarcoplasmic or 147 μg myofibrillar protein fraction was loaded on a lane of a gel. For Gel 2, 88 μg of sarcoplasmic or 135 μg myofibrillar protein fraction was loaded on a lane of a gel. After electrophoresis, fixation was carried out in 50% ethanol (v/v), 10% acetic acid (v/v) for 30 min followed by colloidal Coomassie staining [44]. Gels were destained with Kimwipes (Kimberly-Clark) in Milli-Q water under gentle shaking. High relative molecular mass (M_r) region in the myofibrillar fraction was separated via a second procedure on a 5% T Criterion Tris–HCl precast gel (Bio-Rad) with upper limits of voltage, current and power set to 200 V, 5 mA and 1 W, respectively for 14 h. Fixing, staining and destaining were performed as described above.

2.5. *In-gel trypsin digestion*

2.5.1. *For sarcoplasmic and myofibrillar fractions separated by 4–20% T gels*

For Gel 1, 15 gel sections of approximately equal length (about 5 mm) were excised from each of four gel lanes (duplicate for both sarcoplasmic and myofibrillar fractions) (Fig. 1). For Gel 2, three gel sections of approximately equal length (about 4 mm) were excised from the low M_r region of each sarcoplasmic and myofibrillar fraction in duplicate (see Fig. 1 in Ref. [45]). Each gel section was split perpendicularly into two slices; one slice was used for a LC–MS/MS analysis whilst the other was stored at −80 °C as a back-up. In-gel digestion was performed based on Deb-Choudhury et al. [46] with minor modifications. Briefly, each gel slice was diced into 1–2 mm × 1–2 mm pieces and destained. The gel pieces were briefly washed twice with ammonium bicarbonate solution after reduction and alkylation. After dehydrating with 100% ACN and drying in a vacuum-centrifuge (Labconco, Kansas City, MI, USA), each sample was digested with 150 or 200 ng of sequencing grade trypsin (Promega, Madison, WI, USA) for 16 h. Tryptic digests were extracted subsequently with 10% ACN in 50 mM ammonium bicarbonate, 50% ACN/1% formic acid and finally

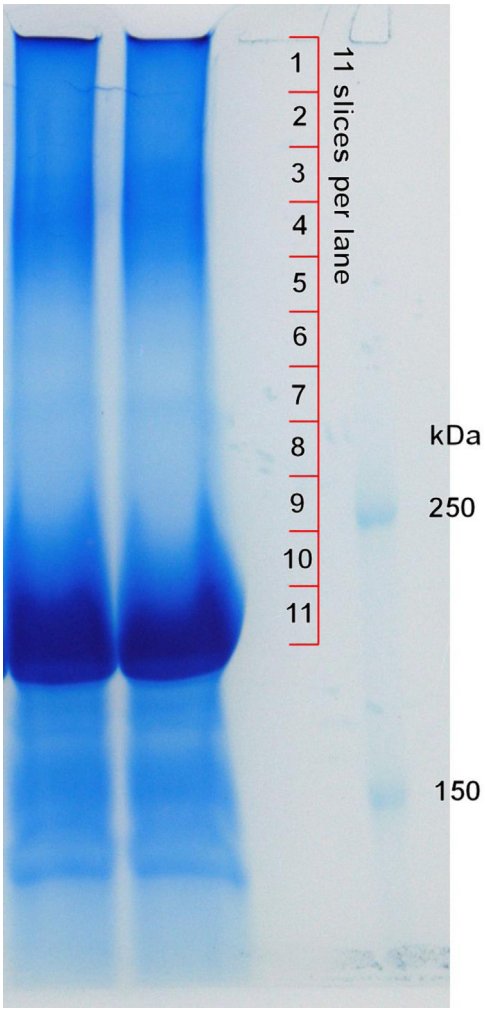

Fig. 2 – SDS-PAGE separation of the myofibrillar fraction across the high M_r region. The marks on the right hand side of the image indicate the approximate position of the gel lanes sliced for proteomic analysis.

80% ACN. Pooled extracts for each sample were dried in the vacuum centrifuge and stored at −80 °C until further analysis.

2.5.2. *For myofibrillar fraction separated by 5% T gel*

The duplicate gel lanes of the myofibrillar fraction in the 5% T gel, spanning from the top of the gel to the myosin heavy chain region, were divided into 11 sections of approximately equal length (about 5 mm) (Fig. 2). The in-gel digestion steps were the same as described in Section 2.5.1.

2.6. *In-solution trypsin digestion of the SDS-insoluble pellet*

Approximately 80 μL of the pellet homogenate (Section 2.3) was spun at 16,000 × *g* for 25 min at 15 °C. The supernatant was removed and the remaining pellet was washed with LC–MS grade water and centrifuged at 16,000 × *g* for 2 min at room temperature. This water wash was repeated, followed by two washes with acetone and one wash with methanol. The remaining pellet was air-dried and kept at −20 °C. Dry pellet was resuspended in 55 μL of 10 mM DTT in 8 M urea and 100 mM ammonium bicarbonate, pH 8 [47] in a sonication bath kept below 25 °C for 10 min. Five microliter of 50 mM TCEP was added to the samples and shaken for 2 h at room temperature. Alkylation was conducted under shaking for 30 min in 25 mM iodoacetamide [47] at 25 °C in the dark. To quench the remaining iodoacetamide, TCEP was added to a final concentration of 12.5 mM with gently shaking at 25 °C for 1 h [modified from 48]. For digestion, 3.3 μg of sequencing grade trypsin and 10% ACN in 50 mM ammonium bicarbonate were added to the sample to dilute the urea concentration to less than 1 M. Sample was shaken at 37 °C for 17 h, followed by an addition of 1.7 μg of the trypsin solution and 4 h of incubation at 37 °C [modified from 47]. Insoluble materials were pelleted down by centrifugation at 16,000 × *g* at 4 °C for 5 min. The supernatant was dried down in the vacuum centrifuge and stored at −20 °C until analysis.

2.7. *LC–MS/MS*

2.7.1. *Gel samples*

Tryptic digests were re-suspended in 50 μL of 5% ACN in 0.1% formic acid and then further diluted with 0.1% formic acid as required. The final concentration of ACN for each sample was always kept ≤2%. Stain intensity was quantified by ImageJ v1.45s (Rasband, National Institutes of Health, USA). Samples from very weakly stained gel sections were re-suspended in 40 μL (applied exclusively to the low M_r region gel slices of the 4–20% T Gel 2) or 50 μL of 0.1% formic acid. The reconstituted samples were spun down at 16,000 × *g* for 5 min at 4 °C and the supernatant was applied for LC–MS/MS.

LC–MS/MS was carried out on a Bruker nano-Advance ultra-high performance liquid chromatography (UHPLC) (Bruker Daltonics, Bremen, Germany) coupled to a Bruker amaZon Speed ETD ion trap mass spectrometer. For a single LC–MS/MS run of each sample, 5 μL (out of 50 μL) was loaded on a C18 trap column (Capillary UHP trap Magic C18AQ, particle size 3 μm, pore size 200 Å, ID 300 μm, Bruker Michrom) at a flow rate of 10 μL/min. The trap column was switched in line with a reversed-phase analytical column (Bruker Michrom Magic C18AQ, particle size 3 μm, pore size 200 Å, ID 0.1 mm, length 150 mm). The loading and desalting solvent used was the same as mobile phase A (0.1% formic acid). The reverse phase elution gradient was 0–45% mobile phase B (98% ACN/0.1% formic acid) in 45 min at a flow rate of 800 nL/min. Between runs, the column was reconditioned by increasing mobile phase B to 95% in 1 min, holding for 6 min and brought down to 0% B in 1 min. The column was equilibrated for 7.5 min in mobile phase A.

Ions were analysed by the mass spectrometer in positive ion mode using automatic MS/MS data acquisition. The maximum ion accumulation time for the ion trap was set to 20.00 ms. Scan range was set to 310–1400 *m/z*. The absolute threshold for precursor ion selection was 100,000. Collision-induced dissociation (CID) was used to fragment precursor ions. A maximum of four MS/MS spectra were acquired after each MS scan. Exclusion time for a precursor ion from further experiments was 0.2 min when a maximum of two MS/MS spectra were acquired from that ion.

2.7.2. *In-solution digested sample*

Dried tryptic digest of the SDS-insoluble pellet was reconstituted in 80 μL of 2% ACN in 0.5% formic acid in a sonicating bath kept below 30 °C for 5 min. The sample was then cleaned using a 200 μL, C18 material StageTip (Thermo Scientific). The cleaning procedure was carried out according to the established protocol [49] except more washing with 0.5% formic acid was applied after sample loading. After drying in a vacuum centrifuge, the sample was reconstituted in 50 μL of 2% ACN, 0.5% formic acid solution. For each LC–MS/MS run, 5 μL of the sample was used. Two LC gradients were used: (1) 0–45% mobile phase B in 60 min, 45–95% B in 1 min, held there for 10 min and brought down to 0% in 0.5 min at a flow rate of 500 nL/min; (2) 0–6% mobile phase B in 1 min, 6–31% B in 50.5 min, 31–61% in 9.5 min, 61–95% B in 1 min, held there for 10 min and brought down to 0% in 1 min at a flow rate of 500 nL/min. The column was equilibrated for 8.5 min in mobile phase A. The settings for MS and MS/MS were the same as described in Section 2.7.1.

2.8. *Data analysis*

2.8.1. *Protein identification*

Mass spectra of each LC–MS/MS run (see Table 1 in Ref. [45]) were converted into a peak list (mgf format) using the DataAnalysis v4.0 software (Bruker Daltonics). Peak lists were separated into the following groups: (1) the sarcoplasmic fraction; (2) the myofibrillar fraction separated on the 4–20% T gels; (3) the myofibrillar fraction separated on the 5% T gel; and (4) the SDS-insoluble pellet, and merged by an in-house programme into four mgf files. These four MS/MS data sets (peak lists) were imported to the ProteinScape v3.1.0 proteomic data repository/bioinformatic software (Bruker Daltonics).

Protein identification procedure was based on Clerens et al. [50] with some modifications. For each of these mgf files, two types of sequence database searches were carried out against each of the two sequence databases: (1) a database of 23,220 protein sequences derived from BGI Shenzen-predicted gene models of the sheep genome version 3 (Oar v3) [51; see http://www.livestockgenomics.csiro.au/sheep/oar3.1.php for details] and (2) the NCBInr *Ovis aries* protein sequence database (December 17, 2012; 25,926 sequences). The two types of sequence database searches were: (1) standard database search using a local Mascot server version 2.4; (2) the same Mascot algorithm implemented with Mascot Percolator using Percolator v1.14 [52,53].

The search parameters for Mascot database searches without Mascot Percolator were: Enzyme specificity was set to semi-trypsin with up to two missed cleavages; for gel samples, Fixed modification was propionamide (C) and Variable modifications were deamidation (NQ), methylation (DE) and oxidation (M); for SDS-insoluble pellet sample, no Fixed modification was used whereas Variable modifications were carbamidomethyl (C), deamidation (NQ), hydroxylation (KP) [31] and oxidation (M); Monoisotopic; MS error tolerance, 0.3 Da; MS/MS error tolerance, 0.6 Da. Each protein identification was required to have at least one peptide with a Mascot score greater than the Mascot Identity Score at a Significance Threshold of $p < 0.05$. Peptide rank cut-off was set to three. Peptides with scores ≤25 or lengths below six residues were all rejected. A peptide decoy database (reverse sequences) search was performed for each search.

The search parameters for the Mascot Percolator-post-processed Mascot search were the same as described above except: the Mascot Percolator option was activated; any protein identification was required to have at least one peptide with a posterior error probability (PEP, a peptide level significance measure expressed as the probability that the observed peptide-spectrum match (PSM) is incorrect [54]) <0.05; Peptide Rank cut-off was set to 1; peptides with a PEP > 0.05 or lengths below eight residues were all rejected.

Mascot search results obtained from the same search strategy against the same sequence database were compiled using the compilation function in ProteinScape. Compiled results were compared and only protein identifications found exclusively in the Oar v3 sequence database derived from the BGI Shenzen-predicted gene models were retained for sequence annotation and updating the in-house sequence database. All entries with an identifier/name corresponding to keratin, hornerin, trypsin or macroglobulin were excluded from further analysis. These Oar v3 protein sequences were searched against the public NCBInr using NCBI BLAST to find similar sequences. Multiple sequence alignment was conducted using ClustalW [55] to assess sequence completeness. As a result, a "representative sequence" sourced from public protein sequence databases was assigned to each protein sequence of interest for naming and retrieving Gene Ontology (GO) annotations if applicable [45]. Annotated protein sequences were curated using CD-HIT-2D [56,57] against the NCBI *O. aries* protein sequence database (August 27, 2013; 30,406 sequences) [45]. Sequences that remained after curation were merged with the NCBI *O. aries* protein sequences (*NCBI Taxonomy: 9940*; August 27, 2013) and the in-house sheep protein sequences [50] to create a combined database for the final Mascot search. These Oar v3 sequences along with their corresponding representative sequences and their proposed names are listed in Supplementary Data 1a of Ref. [45].

The mgf files previously used for updating the in-house *O. aries* sequence database were searched against this updated combined database using the Mascot and post-processed with Mascot Percolator. Search parameters were identical to those of the Mascot Percolator-post-processed searches shown above except carbamidomethyl (N-terminus) was included in the Variable modifications for the in-solution digested sample, carbamidomethyl (N-terminus). This Variable modification was included to assess whether over-alkylation by iodoacetamide has taken place during the preparation of this sample. Nevertheless, the in-house database augmentation was not repeated for the sake of this additional Variable modification because we found the Mascot search results including the modification did not identify any new unique peptides that map to a novel protein sequence derived from BGI Shenzen-predicted gene models for database update.

The two sequence database search results of the myofibrillar fraction (4–20% T and 5% T gels) were combined by the ProteinExtractor algorithm, which was designed to define a minimal protein list that should contain only those proteins (and protein variants) that are distinguishable by the

MS/MS data. Thus, three sets of search results corresponding to the three crude fractions: sarcoplasmic, myofibrillar and SDS-insoluble, were obtained. Proteins were accepted as being robustly identified and validated if each (1) contained at least one unique peptide identified with a PEP < 0.01; or (2) contained at least two unique peptides with a PEP < 0.05. A total minimal protein list was also compiled from all the fractions using ProteinExtractor and Microsoft Excel.

Protein identifications of individual gel slices were based on non-Percolator Mascot searches because the Percolator works best if there are several thousand spectra, which was not applicable to the data sets of these individual slices. Protein identification results of individual gel slices and the search parameter were shown in Supplementary Data 1b, 1c, 1d and 1e of Ref. [45]. These results were used for discussing the gel profile but not for proteome characterisation that involved only the Percolator-post-processed results.

2.8.2. *Function prediction*

Whenever appropriate, a "representative sequence" from UniProtKB (UniProt Knowledgebase) was assigned to a protein identification based on NCBI BLAST search results via the UniProt ID mapping service (http://www.uniprot.org/mapping/) or the batch retrieval tool at the Protein Information Resource (PIR) [58,59]. Only representative sequences that met the following criteria were used for function prediction: (1) for "molecular function" aspect, the query protein shall exhibit max bit scores >245, Expectation values (*E*-values) ≤1.0E−62 [60], query sequence coverage ≥60% and max identity >70% [61]; or query sequence coverage ≥75%, max identity >80% [61] regardless of *E*-values and bit scores (for shorter sequences), compared to its corresponding representative sequence. For "cellular component" aspect, only the representative sequences to which the query proteins exhibited query coverage ≥75% and max identity >80% were accepted [61]. Representative UniProtKB that met the criteria were submitted to QuickGO [62] for obtaining GO Slims associated to these entries and categorising the data retrieved. Hierarchical relationship between GO terms was resolved using SUPERFAMILY [63; http://supfam.cs.bris.ac.uk/SUPERFAMILY/cgi-bin/go.cgi, 64]. Protein identifications that did not have a UniProtKB representative sequence or did not map to any GO annotations of molecular function or cellular component aspect were submitted to InterProScan v4.8 [65,66] for function prediction instead.

3. Results and discussion

3.1. *SDS-PAGE protein profile*

The protein profiles of the sarcoplasmic and the myofibrillar fractions on the 4–20% T gel are shown in Fig. 1. The other 4–20% T gel, from which the low M_r region gel slices were excised, is shown in Fig. 1 of Ref. [45]. The two fractions exhibited distinct banding patterns that were different from each other. The difference in M_r distribution of abundant proteins between the two fractions was particularly apparent. For the sarcoplasmic fraction, abundant proteins found in the most intensely stained region (Fig. 1, ~slices 10–11) included M-type creatine-kinase, mitochondrial malate dehydrogenase, L-lactate dehydrogenase A chain isoform 1 and the glycolytic enzymes fructose-bisphosphate aldolase A isoform 1, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase 1, phosphoglycerate mutase 2 and beta-enolase. For the myofibrillar fraction, abundant proteins found in the heavily stained regions (Fig. 1, ~slice 4–5; ~slices 10–11) included different myosin heavy chain and actin isoforms as well as tropomyosin α and β chains. The patterns of the relatively intense bands were similar to the sarcoplasmic and myofibrillar fractions reported in 48 h post-mortem porcine *longissimus thoracis et lumborum (LTL)* muscle [67].

Skeletal muscle is known to contain cytoskeletal proteins with high M_r such as titin and nebulin [68]. Therefore, we attempted to separate the high M_r region (several hundred kDa) of myofibrillar fraction further using a lower % T gel and apply LC–MS/MS analysis to the region. As shown in Fig. 2, no defined high M_r bands were observed. The lack of a clear nebulin band in 48 h post-mortem sheep *LTL* has also been found to be the case elsewhere [69]. Out of the 22 gel slices from this gel, only 9146 MS/MS spectra were obtained (see Table 1 in Ref. [45]). This number of MS/MS spectra acquired is less than expected. Nevertheless, the standard Mascot search results (see Supplementary Data 1d in Ref. [45]) showed that titin (predicted molecular mass 3812.6 kDa) was mainly found in slices 1–4; nebulin (predicted molecular mass 772.7 kDa) was mainly found in slices 4–6 (Fig. 2) whereas filamin-C (predicted molecular mass 275 kDa) predominantly in slices 7–9 (Fig. 2); a peptide LDVTEPSVVFAK mapped to a high molecular mass protein similar to Obscurin isoform IC, partial (predicted molecular mass 892.2 kDa) was only identified in slices 1, 3 and 4 of one of the replicates with ion scores (not Mascot Percolator scores) of 61.62, 50.29 and 50.74, respectively.

3.2. *Lamb meat proteins*

A total of 388 ovine-specific proteins were identified from the 48 h post-mortem lamb *longissimus lumborum* samples through the use of shotgun proteomic approaches followed by the iterative protein identification process and PSM validation with Mascot Percolator. The number of proteins identified in each fraction is summarised in Fig. 3. Identified proteins and the identified peptides matched to them are presented in Supplementary Data 2 of Ref. [45].

The theoretical M_r of the identified proteins ranged from 5.7 kDa (PREDICTED: pentatricopeptide repeat-containing protein 1, mitochondrial-like isoform 3) to more than 3000 kDa (PREDICTED: titin). Interestingly, a large protein "similar to Obscurin isoform IC, partial [*O. aries*: OARv3]" was identified in both the 4–20% T gel and 5% T SDS-PAGE samples but with only one and the same unique peptide sequence LDVTEPSVVFAK. Obscurin (~800 kDa) is a sarcomeric protein that interacts with titin [70] and is known to determine the architecture of sarcoplasmic reticulum [71]. A quarter of the total proteins (108 out of the 388 proteins) were identified by a single unique peptide at the level of individual fractions but all the matching peptides had a Mascot Percolator score >20.0 (i.e., PEP < 0.01). Annotated spectra for these protein identifications are reported in Supplementary Data 3a-3c of Ref. [45].

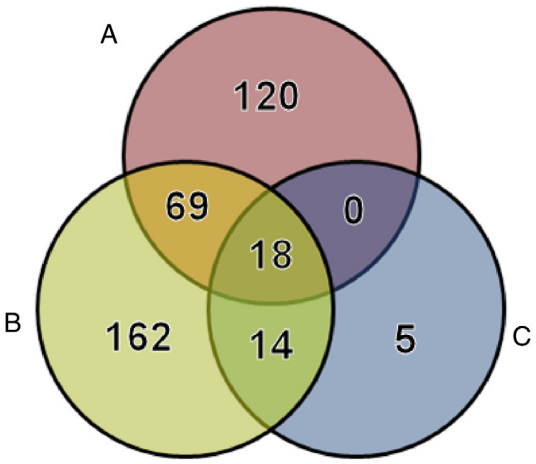

Fig. 3 – Number of proteins identified in the three crude fractions. (A) Sarcoplasmic fraction; (B) myofibrillar fraction (containing both 4–20% T and 5% T gel samples); (C) SDS-insoluble pellet.

The GO Slims (http://www.ebi.ac.uk/QuickGO/GMultiTerm) annotation of the total protein list via representative sequences (see Supplementary Data 4 in Ref. [45]) indicated that proteins from a broad range of molecular function categories were found in the lamb meat (Fig. 4). A relatively larger proportion of the proteins was found to have functions relating to the GO terms protein binding, ion binding, nucleotide binding and/or oxidoreductase. These features are consistent with the nature of skeletal muscle as being a highly metabolically active tissue in an animal. With respect to subcellular localisation, mitochondrion was the largest category, containing around one quarter of the proteins, followed by cytosol, nucleus and cytoskeleton (Fig. 5). Details of GO annotations for the representative sequences are given in Supplementary Data 5–8 of Ref. [45].

Out of the 388 proteins, 39 remained unassigned with molecular function and/or cellular component annotations. These proteins were separately analysed with InterProScan, a publicly available tool that evaluates a given protein sequence for protein signatures (e.g., protein domain families) of a collection of signature databases [65]. InterProScan analysis results are given in Supplementary Data 4 of Ref. [45]. All these proteins, except the unnamed/uncharacterised proteins, have an informative name that is supported by the results. For

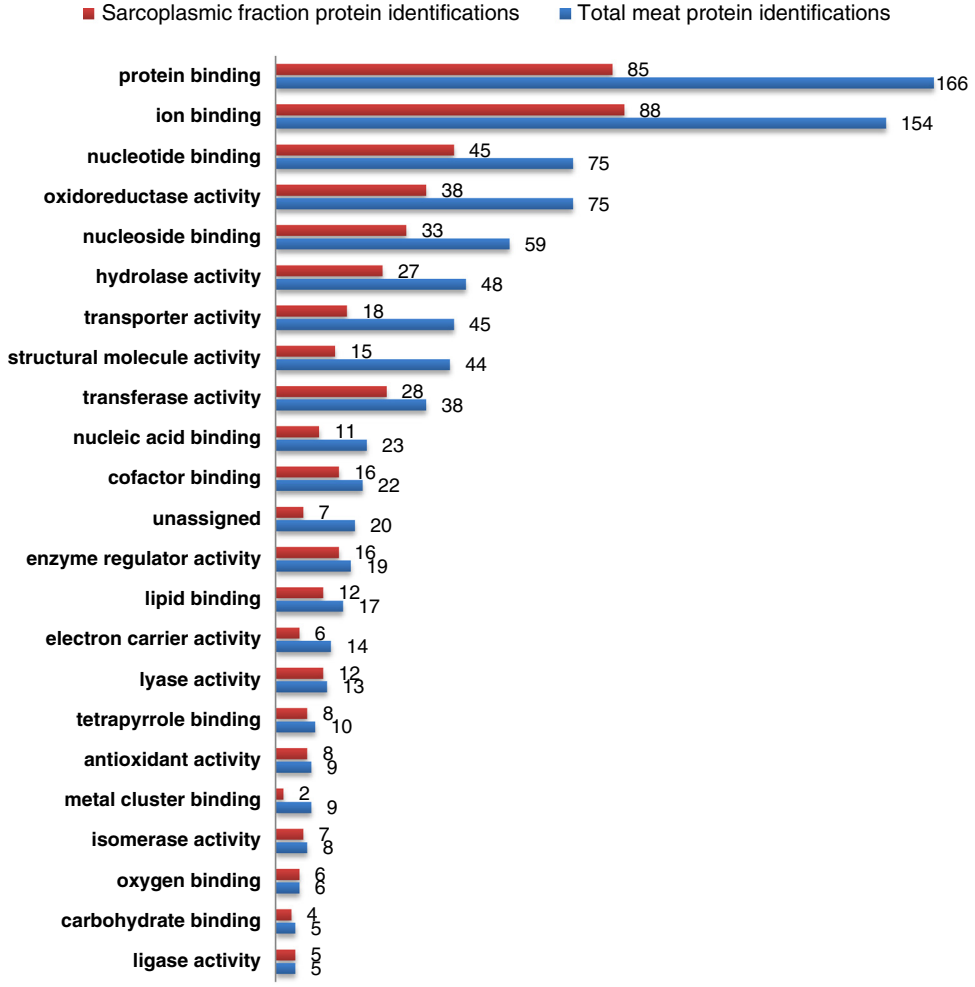

Fig. 4 – Comparison between the molecular function GO Slim assignments of the total protein identifications and that of the sarcoplasmic fraction.

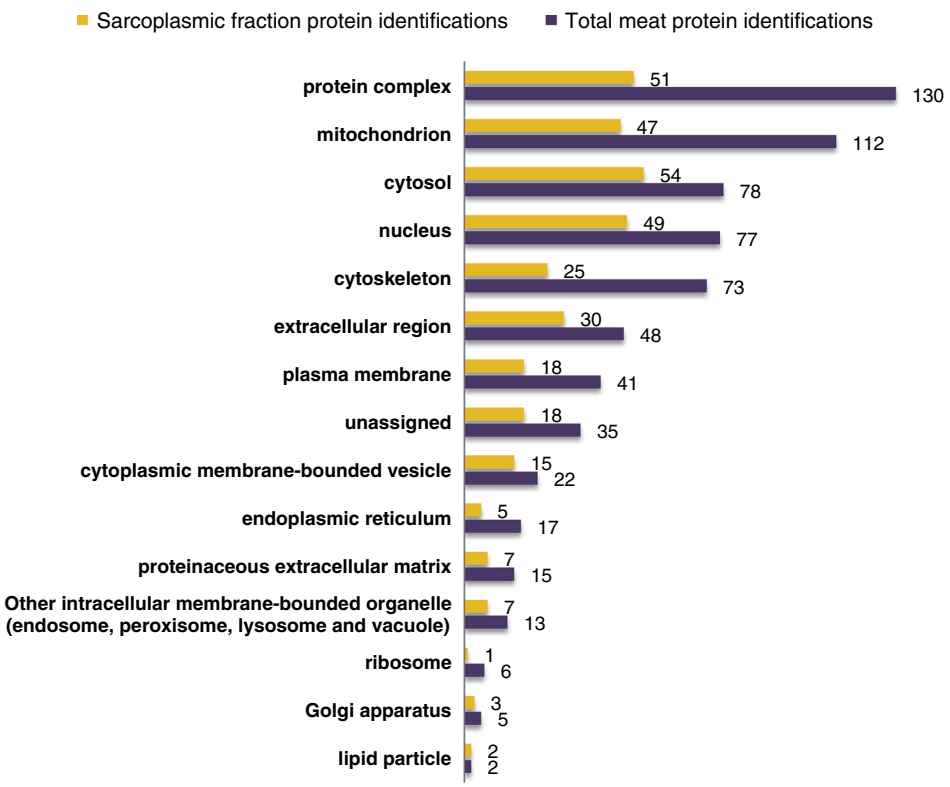

Fig. 5 – Comparison between the cellular component GO Slim assignments of the total protein identifications and that of the sarcoplasmic fraction.

example, the protein PREDICTED: myosin-binding protein H [*O. aries*] (gi|426239359) appears to contain fibronectin type III domain that has been found in the human myosin-binding protein H (UniProt: Q13203). Predicted potential molecular functions (in GO Slim terms) of these proteins are summarised in Fig. 6 based on manual interpretation of this InterProScan analysis.

We have identified 207 proteins (see Supplementary Data 2 in Ref. [45]) in the sarcoplasmic fraction, which contains protein soluble in water at low ionic strength. The majority of the enzymes that carry out glycogen metabolism, glycolysis and tricarboxylic acid cycle were found with at least one isoform or subunit. Based on the available GO annotations, it appears transferases, lyases and proteins involved in enzyme regulation, co-factor binding, lipid binding and antioxidant activity were mainly found in the sarcoplasm of the lamb meat (Fig. 4). Several sarcomeric proteins, including titin, actin, filamin, alpha-actinins, myomesins, myosin-binding protein C, myosin heavy chain and myosin light chains [72,73], were also found in the sarcoplasmic fraction. This observation might be explained, at least in part, by the fact that some of these proteins have been shown to undergo post-mortem degradation [74].

In the myofibrillar fraction, 263 proteins were identified (see Supplementary Data 2 in Ref. [45]) including various sarcomeric proteins. Some groups of proteins were found exclusively here, including various membrane proteins such as transmembrane protein 109, voltage-dependent anion-selective channel protein 2 isoform 2, several NADH dehydrogenase and ATP synthase subunits, reticulon 2; cytoskeletal proteins such as several different tubulin chains and extracellular matrix proteins type VI collagen α-1 chain-like and α-3 subunits. Several sarcoplasmic proteins, e.g., glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, glycogen myophosphorylase and pyruvate kinase isozymes were also found in the myofibrillar fraction. Their presence may be caused by the residual aqueous phase retained in the pellet after the extraction of sarcoplasmic fraction. This was realised from the onset as the pellet was purposely left unwashed to avoid losing proteins of low abundance. However, it is possible that there are interactions of glycolytic enzymes with other structural components of myofibrils [75], e.g., phosphofructokinase [76]. Concurrently, the tryptic peptides mapped to phosphofructokinase were found solely in the myofibrillar fraction in this study.

Although meat is derived from skeletal muscle, the physiochemical changes occurred during skeletal muscle to meat conversion such as gradual deletion of available energy, drop in pH and increase in ionic strength, have a pronounced impact on numerous proteins in the muscle cell [74]. Examples include post-mortem protein degradation [77,78], increased level of oxidation [79,80] and phosphorylation [36,67] of specific muscle proteins during post-mortem storage. Therefore, whether post-mortem changes have a role in reducing the abundance and/or confound the identification of these proteins may require further study.

In the SDS-insoluble pellet fraction, 37 proteins were identified (see Supplementary Data 2 in Ref. [45]). However, a

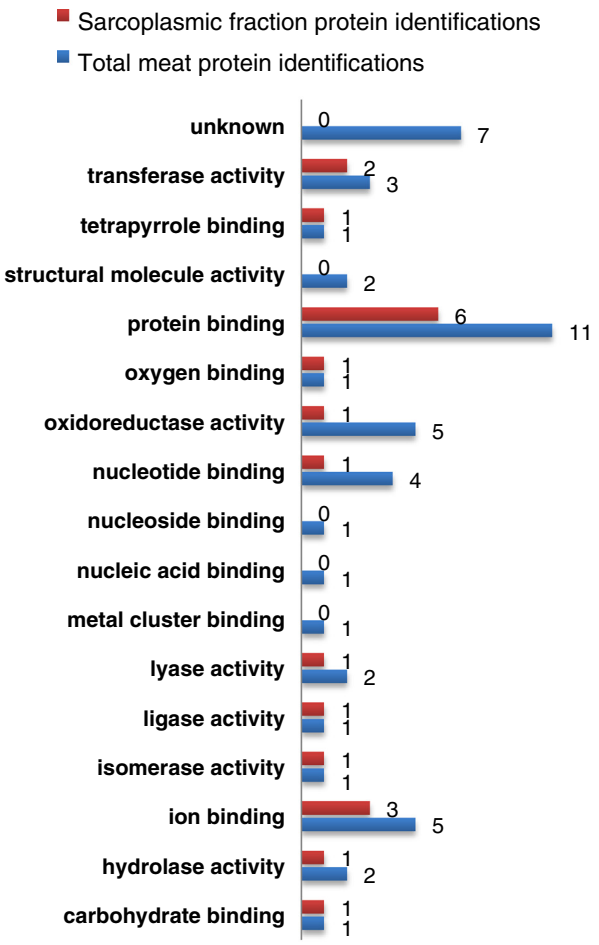

Fig. 6 – Summary of the predicted potential molecular functions of the proteins with unassigned GO terms (see Section 3 for details) based on manual interpretation of the InterProScan analysis results.

majority of them (86%) were also found in the myofibrillar fraction. The remaining five proteins were different types of collagen α-chains. The highly abundant myofibrillar proteins titin and myosin-1 were among the top five proteins with the highest number of unique and/or non-redundant peptides in this fraction. By including carbamidomethyl (N-terminus) as a Variable modification in the final Mascot Percolator-post-processed Mascot search, we found 14.7% of the peptide identifications contained a carbamidomethyl N-terminus and they were mostly from type I and II collagens (see Supplementary Data 2 in Ref. [45]). Curiously, all these peptides with "a carbamidomethyl N-terminus" were all semi-tryptic with a glycine residue in the P1 position of the "non-tryptic cleavage" terminus (see Supplementary Data 2 in Ref. [45]). A glycine residue exhibits a very close monoisotopic mass (57.02 Da) to that of carbamidomethylation (57.03 Da). Also, by randomly mapping some of these peptide sequences to their corresponding protein sequences (e.g., EPGPVGAVGPAGAVGPR in collagen alpha-2(I) chain), we found the peptides were tryptic if the glycine residues were shifted from the P1 position to the P1′ position. Thus it is possible that these non-specific alkylation identifications, at least to some extent in this particular case, may not actually exist even though we cannot prove it based on the results.

LTL is frequently used in meat research [81], including many studies that employ proteomic techniques. Currently, most of them are differential studies aiming to identify only the proteins that exhibit differential abundance, to the best of our knowledge. A few studies focusing on LTL did involve identification of the proteome in their samples, including one for bovine [82] and three for porcine [28,83,84]. They are briefly summarised in Table 1. By comparing the proteins reported by these studies with our results, both similarities and differences were found. For example, the study on porcine LTL exudate proteome [28] identified several enzymes involved in energy metabolism (e.g., phosphoglycerate kinase 1, alpha-enolase and malate dehydrogenase, cytoplasmic) whose ovine analogues were also detected in the sarcoplasmic fraction of our study. Also, while both our and the bovine study [82] identified the abundant skeletal muscle proteins such as myosin-1 (myosin heavy chain 1), troponin T, fast skeletal muscle and glucose-6-phosphate isomerase, some proteins were detected only in the bovine study, e.g., cysteine and glycine-rich protein 3.

However, it is uncertain whether all these observed differences actually arise from species differences for two main reasons. Firstly, the proteomic workflows employed by these studies, e.g., gel-free 2D-LC–MS/MS or 2-DE in conjunction with MALDI-TOF/TOF and/or LC–MS/MS, were different from those used here. The differences could lead to identification of non-overlapping sets of proteins between approaches, as highlighted in Raddatz et al. [39] in the case of murine heart muscle proteome. Secondly, species-specific database coverage at the time when the studies were conducted might differ. For example, the bovine *longissimus thoracis* proteomic study carried out by Bjarnadóttir et al. [82] used a bovine database whereas the porcine LTL phosphoproteomic study published recently by Huang et al. [83] utilised the mammalian database because the porcine database was not complete at that time.

3.3. *Criteria for protein identification*

In this study we accepted proteins identified by a single unique peptide as Chen et al. [85] reported in their proteomic characterisation of silkworm larval gonads for a similar rationale: the identification of a single unique peptide often provides sufficient evidence to conclude the presence of a product of a certain gene, as stated by Schirmer et al. [86] and Nesvizhskii and Aebersold [87]; further, in a typical shotgun proteomic experiment, more than 30% of all detected proteins are identified by a single peptide, including many low molecular weight and low abundance proteins [87]. Proteogenomic studies performed on *Shewanella* species have indicated complete exclusion of proteins with a single identified peptide often leads to loss of a large number of protein identifications (20–25% of all expressed proteins) [88]. In order to minimise false-positively identified proteins while retaining single-peptide identifications, a stringent peptide scoring system administered by Mascot Percolator was employed as a validation step for our final database search results. Details of these unique peptides are reported in Supplementary Data 2 of Ref. [45].

Table 1 – Brief summary of proteomic studies that involved proteome identification specific to LTL muscle.

Authors	Species	Main purpose of the study	Protein extraction solution composition	Separation at protein level	Separation at peptide level (for the digested protein samples)	Data acquisition technology (e.g., ESI-MS/MS, protein microarray, etc.)	# protein identified
Bjarnadóttir et al. [82]	Cattle	To find proteins with changed abundance between animals producing tender or tough meat using iTRAQ and 2-DE.	Chaotrophes/ denaturants, zwitterionic detergent and reducing agent	2-DE for the gel-based workflow	2D-LC for the gel-free iTRAQ work flow	ESI-MS/MS	iTRAQ: out of the two iTRAQ sets, 115 and 143 proteins were found. The 100 proteins that were identified in both sets were reported. 2-DE: 10/13 proteins showing significant change were identified and reported.
Hornshøj et al. [84]	Pig	To compare the transcript profiles and protein expression profiles of porcine heart and skeletal muscles using 454 sequencing, cDNA microarray and iTRAQ.	Tris, EDTA and sucrose (see the paper referred in Ref. [84] for details)	No	2D-LC	ESI-MS/MS	The proteins corresponding to 354 UniGene IDs were identified. However only 148 of the UniGene IDs that mapped to the transcriptomic experiments were reported.
Di Luca et al. [28]	Pig	To generate a 2D DIGE-based proteome map of porcine muscle exudates collected at different post-mortem time points from three phenotypes differing in water-holding capacity.	N/A	Muscle exudates collected and 2-DE used for preparative gels	RP-LC for ESI-MS/MS	MALDI-TOF/TOF and ESI-MS/MS as required	Eighty-nine spots corresponding to 122 proteins/fragments were identified and reported.
Huang et al. [83]	Pig	To identify and characterise the phosphorylation sites that change in post-mortem muscle using the quantitative MS-based analysis.	Chaotrophes/ denaturants, zwitterionic detergent, reducing agent, Biolyte pH 3–10, protease and phosphatase inhibitors	No	For non-phosphorylated peptides: titanium oxide chromatography followed by hydrophilic interaction liquid chromatography (HILIC) and then reverse phase (RP)-LC For phosphopeptides: titanium oxide chromatography and sequential elution from immobilised metal affinity chromatography for enrichment; HILIC and/or RP-LC for separation (see [83] for details)	ESI-MS/MS	In total, 305 unique proteins were identified and reported.

As opposed to false discovery rate (FDR) that measures the error rate associated with a collection of PSMs, PEP measures the probability of error for a single PSM [54]. Thus, PEP = 0.01 for a peptide means that the chance of a wrong peptide identification would be 1% in the worst-case scenario [89]. With respect to the FDR of the final database searches of the four MS/MS datasets, the values ranged from 0.36% to 1.64% when the PEP threshold was set to <0.05 (see Supplementary Data 4 in Ref. [45]). With a PEP threshold of <0.01, the FDR were all below 0.2%. This low FDR associated with a Mascot Percolator score >20.0 showed good confidence of the protein identifications based on a single unique peptide.

Currently, more than two thirds of *O. aries* protein sequences in NCBI were of a predicted nature (as of August 27, 2013). Unsurprisingly, most of the proteins identified in this study were based on these predicted sequences. Out of the total 388 protein identifications (according to the total protein list presented in Supplementary Data 2 of Ref. [45]), 308 entries were predicted by NCBI (those with an accession # beginning with XP) and six entries derived from BGI Shenzhen-predicted gene models (those with an in-house gi # starting with 1999). Among the identified NCBI-predicted protein entries, 30 of them have a title prefixed with "PREDICTED: LOW QUALITY PROTEIN". This prefix indicates the gene models from which these protein sequences are derived contain insertions, deletions or frameshifts yet they have a strong unique hit in the SwissProt database or seems to be orthologues of known protein-coding genes [90]. Therefore, the proteomic experiments provided evidence for the existence of these predicted protein sequences and therefore their corresponding gene models, including those that have been known to have potential defect(s).

3.4. *Criteria for Gene Ontology annotation*

As mentioned above, the majority of the identified proteins were predicted ones that lack functional annotations, e.g., GO terms, in public databases. On account of that, a simple sequence-based inference approach was taken to transfer GO annotation from UniProtKB entries through NCBI BLAST based on the set criteria (as stated in Materials and Methods). Literature findings on the correlation between GO terms and sequence similarity [60,61] were taken to devise these criteria.

Although higher sequence similarity increases confidence in function annotation transfer, there is no sequence similarity threshold that ensures that two proteins share the same function [91]. For example, Clark and Radivojac [92] reported that even at 100% global sequence identity, perfect transfer of molecular function and biological processes in terms of GO terms is not always observed. In addition, the GO annotations of the representative sequences from which we derived the functional annotations vary in their evidence codes (see http://www.geneontology.org/GO.evidence.shtml for definitions). Most of these evidence codes were of "Inferred from Electronic Annotation" (IEA) (see Supplementary Data 5–8 in Ref. [45]), which is computationally derived and has not been manually curated. Taken together, protein function prediction inferred from sequence similarity is a simple way to impart useful hints on what kind of protein these identified "gene model-derived proteins" might be but their actual functions can only be verified with biological data.

4. Conclusions

This study reports the first in-depth proteomic characterisation of lamb meat derived from *longissimus lumborum* muscle. Meat proteins across a wide range of molecular masses, solubilities and functions were identified with high statistical confidence. In addition, the results confirmed the presence of at least 300 predicted protein sequences from the Oar v3 database. The protein catalogue presented here will contribute to the current understanding of the global protein composition of lamb skeletal muscle/meat and serve as a reference for the lamb meat industry in New Zealand and other countries. The proteome data could help pave the way towards better understanding of the nutritional value of lamb proteins. As a step towards a comprehensive proteome map of all lamb muscle/meat types, it also provides better understanding of the raw product, allowing product differentiation and market segmentation. The proteome data could also support efforts towards the discovery of bioactive/functional sequences that might hold potential as value-added ingredients or products to the meat industry.

Conflict of interest

The authors have declared no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

The authors would like to thank Jessica Gathercole, Grant Smolenski and Guojie Wu for critical review of this manuscript. Funding for this project was provided by AgResearch Core Funding, Lincoln University and the Biomolecular Interaction Centre as part of the Red Meat CombiFoods Programme (MBIE reference # C10X1005; AgResearch Contract # A19119(A)).

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