PROTEIN PROFILE DIFFERENCES IN LONGISSIMUS MUSCLE OF TENDER AND TOUGH LAMB AT 1 AND 5 DAYS OF AGEING

D. Jeong[2,3,4], M.I. Knight[1,2], I.H. Hwang[3], B.Y. Park[4], D.L Hopkins[1,5] and M.B. McDonagh[1,2*]

[1]Australian Cooperative Research Centre for Sheep Industry Innovation, Armidale, NSW 2350, Australia.

[2]Department of Primary Industries Victoria, Biosciences Research Division, Bundoora Victoria 3083, Australia

[3]Department of Animal Resources and Biotechnology, Chonbuk National University, Jeonju , 561-756, Republic of Korea

[4]Quality Control and Utilization of Animal Products Division, National Institute of Animal Science, RDA, Suwon, 441-350, Republic of Korea

[5]NSW Industry & Investment (Primary Industries), Centre for Sheep Meat Development, Cowra, NSW 2794, Australia

[*]Corresponding author (phone: +61-3-9479-3821; fax: +61-3-9479-3618; e-mail: Matthew.McDonagh@dpi.vic.gov.au)

Abstract—**This experiment aimed to determine changes in the proteomic profiles from m. *longissimus thoracis et lumborum* (LL) of lambs selected as extremes of tenderness and toughness from a population group. To achieve the aim, two different comparisons were performed. The first comparison investigated changes in protein profiles of tender and tough LL muscle samples collected after Day 1 of ageing (Experiment 1; n = 9 for each group). Comparison 2 investigated changes in protein profiles of tender and tough LL muscle samples collected after 5 days of ageing (n = 9 for each group). The average Warner Bratzler shear force (WBsf) readings for the tender and tough LL muscle samples in Experiment 1 was 3.27kg(pH5.54) and 6.30kg(pH5.60), respectively. Whilst, Experiment 2, the tender and tough LL muscle samples had an average WBsf readings of 2.48kg(pH5.62) and 3.16kg(5.52) at Day 5, respectively. All samples used in Experiment 2 had an initial WBsf reading of 4.5kg at Day 1 ageing. Proteomic profiles were examined by 2D-Fluorenscence Difference In-Gel Electrophoresis (2D-DIGE) and proteins with altered relative abundance between groups were identified by MALDI TOF/TOF mass spectrometry. In experiment 1, 25 proteins showed altered relative abundance between the tender and tough LL muscles samples at Day 1 of ageing. These proteins were primarily metabolic enzymes involved in glycolysis and heat shock proteins involved in stress responses within the cell. In experiment 2, 22 proteins had altered relative abundance in the LL muscle of lamb after ageing for 5 days. In this experiment, the differentially modified proteins were also predominantly metabolic enzymes.**

Index Terms—**Lamb, Tenderness, 2D-DIGE, Minimal dye, LL muscle.**

I. INTRODUCTION

According to a study by Savell *et al.* (1987), meat tenderness and to a lesser extent, flavour and juiciness, affect the decisions of consumers when purchasing meat. Intrinsic meat quality attributes, especially meat tenderness, depend on post-mortem factors associated with meat storage (ageing) and on muscle characteristics developed in the live animal (Hocquette, *et al.,* 2009). When meat is eaten by consumers, meat tenderness is regarded as the most important factor that influences consumers perceptions of eating quality and so understanding the variation in tenderness is important. A good understanding now exists as to the enzymatic groups responsible for improvements in tenderness (Hopkins & Geesink, 2009) and proteomics has provided a new capability to study the change in proteins due to enzymatic action providing the opportunuity to improve our understanding of these changes beyond that documented with one dimensional electrophoresis (eg Hopkins and Thompson, 2002).

There are a number of proteomics papers in the meat science literature that analyse genetic differences in expressed meat quality traits such as meat colour, marbling, meat ageing and tenderness (Jia, et al., 2007; Bendixen, 2005, Hocquette, et al., 2009; Mullen, et al., 2006 and Jia. X., et al., 2006). Recent advances in 2-D gel electrophoresis (2DE) which utilise differential fluorescence-tagging of comparable samples, prior to their multiplexed separation and analysis, has improved the quantitative properties of 2DE based comparative proteomics. This technique, known as 2D-DIGE, has enabled accurate quantitative comparison between proteins on different gels (Timms & Cramer, 2008), but has not been applied to studies of changes in lamb muscle.

This study used 2D-DIGE to identify differentially regulated proteins in tender and tough LL muscle samples based on the initial Day 1 shear force reading (Experiment 1) and tender and tough LL muscle samples based on the final Day 5 shear force readings (Experiment 2).

II. MATERIALS AND METHODS

A. Experimental design

Full experimental details on the lambs have been provided by Hopkins, Stanley, Martin, Ponnampalam, & van de Ven (2007a). Briefly a 4 × 2 x 2 factorial experiment was conducted in which lambs from 4 sire groups were weaned at either 20 or 30 kg live weight and then either maintained at that weight for 55 days and then re-alimentated or fully fed from weaning until slaughter. After weaning the lambs grazed a combination of lucerne and pasture grasses and were fed supplements as detailed by Hopkins *et al.* (2007a). From these lambs a subset of lambs were selected. In experiment 1 two groups of lambs which had a different initial shear force at Day 1 of ageing - tender samples, 9 animals, WBsf 3.27kg and tough samples, 9 animals, WBsf 6.30kg were compared. In experiment 2 two groups of lambs which had a different final shear force after 5 days of ageing - tender samples, 9 animals, WBsf 2.48kg and tough samples, 9 animals, WBsf 3.16 were compared. All samples used in experiment 2 had an initial WBsf reading of 4.5kg at Day 1 ageing.

B. Sampling and Warner Bratzler meat shear force measurements

All lambs were electrically stunned (head only) and all carcasses were electrically stimulated (800 milliamperes with variable voltage to maintain a constant current, for 34 seconds at 14 pulses/s, 1 millisecond pulse width) post-dressing with a mid-voltage unit. Carcasses were chilled at a mean temperature of 4–5°C. The left side loin was removed with a knife from all carcasses and part of this was divided into 2 portions (cranial and caudal) for shear force testing. Chilled 5 day samples were vacuum packed and held chilled (4-5°C) until preparation and freezing on day 5. Samples of LL were prepared into 65 g blocks and frozen (–20°C) at either 1 or 5 days of ageing for subsequent shear testing. Further details on shear force testing are given by Hopkins, Stanley, Toohey, Gardner, Pethick & van de Ven (2007b).

Approximately 20g of muscle sample was taken from the LL muscle, frozen in liquid nitrogen and ground to a powder form in liquid nitrogen using Mixer-Mill (25Hz, 3 min; Retsch MM400, Germany) and then stored -80°C freezer until analysis.

C. 2D-DIGE and Image analysis

Two hundred milligrams of powdered muscle sample was extracted in 2ml mild detergent buffer containing 40mM Tris, 250mM Sucrose, 5mM $MgCl_2$, 2mM EGTA, 10mM KCl, 0.2%(v/v) Triton X-100 and 40μl of a protease inhibitor cocktail – EDTA-free (Roche, Germany). After resuspenion in mild detergent buffer, the samples were sonicated below 15°C for 10 min and then centrifuged at 40,000 x *g* for 45min at 10°C (Beckman Ultracentrifuge, USA). After centrifugation, 1 ml of supernatant was precipitated in 9 volumes of ice-cold acetone. The protein precipitate was then collected by centrifugation and solubilised in 0.5ml DIGE labelling buffer, pH8.5 containing 7M Urea, 2M Thiourea, 30mM Tris, 1.2% (w/v) CHAPS and 0.4% (w/v) ABS-14.

The protein concentration of each sample was determined using 2-D Quant-Kit (GE Healthcare, USA). Fifty micrograms of protein for each sample was labelled with 200ρmol CyDye DIGE Flours minimal dye (GE Healthcare, USA). Samples were labelled with Cy3 and Cy5 DIGE fluors and an internal standard which consisted of a mixture composed by proteins of all samples was labelled with Cy2 DIGE fluor according to the manufacturer's instruction. Once the labelling reaction was completed, all samples were paired and a rehydration solution containing 7M Urea, 2M Thiourea, 30mM Tris, 1.2% (w/v) CHAPS, 0.4% (w/v) ABS-14, 10mM Acrylamide, 20mM DTT, 5μl DeStreak reagent(GE Healthcare, USA) and 2.25μl Carrier ampolyte reagent pH4-7(GE healthcare) added.

A total of 150ug protein per each pair was loaded onto 24cm-long immobilized pH gradient dry strip (pH range 4-7, GE healthcare), and seperated using Ettan-IPGphor and the Ettan dalt system (GE healthcare) with 12% Acrilamide-bis gel. After 2DE, the fluorescence's of labelled protein in the gels was scanned using a Typhoon™-Trio scanner (GE Healthcare). The overall intensity of the spots was quantified and compared using DeCyder 7.0 software (GE Healthcare) and statistical significance of protein abundance for each protein spot was determined using same program.

D. MALDI TOF/TOF Mass Spectrometry and Protein Identification

Significantly different protein spots ($P < 0.05$) were picked from gels and proteins in gel plugs were digested with trypsin and eluted with a matrix solution (α-cyano-4-hydroxzcinnamic acid) onto a MALDI target plate (Applied Biosystems) using an Ettan Spot Handling Workstation system 2.1(GE healthcare). Peptide mass and fragment masses were obtained in positive ion reflector mode using a MALDI-TOF/TOF mass spectrometer (Applied Biosystems 4700, Applied Biosystems, USA), and peptide masses matched with using the NCBI database and the MASCOT search program (www.matrixscience.com).

III. RESULTS AND DISCUSSION

The protein expression of two tenderness groups were compared in this study. The first group had different initial shear force (day 1 post-mortem) and were classified to tender and tough (Exp. 1). Protein expression was also compared in day 1 aged samples that showed high or low shear force at 5 days post-mortem (Exp. 2). Figure 1 shows a 2DE pattern of protein extracted sample on a pooled standard protein map. For the first experiment there was a significant difference in protein abundance for 19 protein spots ($P < 0.05$) using 2D-DIGE, and of these 16 different proteins were identified (Table 1). The identified proteins were divided into two groups; 1) metabolic enzyme proteins and 2) cellular stress proteins. In the former group, three proteins were detected with higher intensity in the tender meat group compared to the tough meat group after 1 day of ageing. These proteins are phosphorylase (#510), enolase 1(#1642), and malate dehydrogenase 1(#2300). The remaining proteins were detected at higher intensity in the tough meat group.

For the second experiment, where 1 day aged samples from animals classed as tough or tender based on 5 day shear force measurements were studied, 17 protein spots showed altered relative abundance and 15 unique proteins were identified. These 15 proteins were classified into three functional groups, metabolic enzyme proteins, cellular defence/stress proteins and a miscellaneous protein groups (Table 2). These 15 proteins showed significantly higher intensity in the tough group compared to the tender groups ($P < 0.05$).

The majority of proteins in the metabolic enzyme related group from experiment 2 are involved in glycolysis and/or TCA cycle. Hence, we observed significant differences across a range of enzymes that were identified in the first experiment. This suggests that changes in these proteins are related to initial meat tenderness and ageing and/or meat quality at 5 days of ageing. These proteins include those related glycolysis or TCA cycle and/or cellular stress protein. However the proteins altered in abundance of different initial tenderness group was composed metabolic enzyme and heat shock family proteins. These results imply that similar mechanisms are involved in establishing day 1 and day 5 tenderness in lamb. Initial tenderness is either regulated by a combination of metabolic enzyme activity and stress protein responses in post-mortem muscle cells, or proteases that degrade these proteins are important in initial lamb tenderness. With regard to day 5 tenderness, the influence of metabolic enzymes appears more important based on fewer stress proteins showing altered relative abundance between the tough and tender groups.

IV. CONCLUSION

In these experiments the proteome profiles of lamb muscle at 1 day post mortem, from groups with high or low tenderness at 1 and 5 days post mortem were compared. Differences in initial tenderness was associated with changes in the intensity of proteins related to metabolic enzyme activity through the glycolysis pathway and the cellular stress response proteins such as the heat shock family of proteins. Differences in the proteome profiles of samples with high or low tenderness at 5 days of ageing were associated with a similar profile of metabolic enzymes.

ACKNOWLEDGEMENTS

Technical support for this study was provided by Leonie Martin, Tony Markham, Jayce Morgan, Andrew Roberts, Megan Moppett, Tracy Lamb, Geoff Casburn, Brent McLeod, Joe Brunner and Stuart McClelland I&I NSW (Primary Industries), Andrew Slack-Smith and Jason Siddell (University of New England), Peter Allingham (CSIRO), Peter Walker, Matt Kerr, Dete Hasse, Erin Rutty, Paul Eason and Fahri Fahri (DPI, Victoria), Dr Greg Nattrass and Victoria Ferris (SARDI), Mal Boyce and Grace Chan (Murdoch Uni), Dr Robin Jacob (DAWA) and Linden Cowper, Janine Lau, Nathan Sheahan and Colin Cowell (MSA). Testing of samples for shear force was undertaken by the meat science section at the University of New England. The excellent co-operation of Junee Abattoir employees and management is gratefully acknowledged. The study was funded by I&I NSW (Primary Industries), DPI Vic, Meat and Livestock Australia and the Australian Sheep Industry Cooperative Research Centre.

REFERENCES

Bendixen, E. (2005). The use of proteomics in meat science. Journal of Meat Science, 71, 138-149.

Hocquette, J. F., Isabell, C. M., Carine B. C., & Brigitte P. (2009)Functional genomics and now markers for beef production-minireview. Annimal science paper and reports, 27, 273-280.

Hopkins, D.L. and Thompson, J.M. (2002). The degradation of myofibrillar proteins in beef and lamb meat using denaturing electrophoresis – An overview. *Journal of Muscle Foods* 13, 81-102.

Hopkins, D.L., Stanley, D.F., Martin, L.C., Ponnampalam, E.N. and van de Ven, R. (2007a). Sire and growth path effects on sheep meat production. 1. Growth and carcass characteristics. *Australian Journal of Experimental Agriculture* 47, 1208-1218.

Hopkins, D.L., Stanley, D.F., Toohey, E.S., Gardner, G.E., Pethick, D.W. and van de Ven, R. (2007b). Sire and growth path effects on sheep meat production. 2. Meat and eating quality. *Australian Journal of Experimental Agriculture* 47, 1219-1228.

Hopkins, D.L. and Geesink, G.H. (2009). Protein degradation post mortem and tenderisation. In Applied Muscle Biology and Meat Science, pp. 149-173, (Ed Du, M. and McCormick, R.), CRC Press, Taylor & Francis Group, USA.

Jia, X., Hildrum, K. I., Westad, F., Kummen, E., Aass, L. & Hollung K. (2006). Change in enzyme associated with energy metablism during the early post mortem period in longissimus thoracis bovine muscle analyzed by proteomics. Journal of Proteomic Research, 5, 1763-1769.
Jia, X. H., Ekman, M., Grove, H., Faergestad, E. M., Aass, L., Hildrum, K. I. & Hollung, K. (2007). Proteome changes in bovine longissimus thoracis muscle during the early post-mortem storage period. Journal of proteomic research, 6, 2720-2731.
Mullen, A. M., Stapleton, P. C., Corcoran, D. Hamill, R. M.. & White, A. (2006). Understanding meat quality through the application of genomic and proteomic approaches. Meat Science, 74, 3-16.
Timms, J.F & Cramer, R. (2008). Difference gel electrophoresis. Proteomics, 8, 4886-4897.

Table 1. Identification of differentially expressed proteins between meat groups showed different initial shear force by Proteins were classified by function of proteins in skeletal muscle cell following the study of Jia et al.(2007)

Spot no	Protein Description	Accession number	Species	MOWSE	M.Wt.	Protein score C.I%
Metabolic enzyme						
510	Phosphorylase, glycogen, muscle	gi|154426116	Bos taurus	244	97795	100
1255	phosphoglucomutase 1	gi|116004023	Bos taurus	277	61906	
1378	phosphoglucomutase 1	gi|116004023	Bos taurus	440	61906	100
1632	mitochondrial aldehyde dehydrogenase 2	gi|115496214	Bos taurus	434	57185.2	100
1642	enolase 1	gi|87196501	Bos taurus	119	47723	
1823	enolase 3 (beta, muscle), isoform CRA_b	gi|119610782	Homo sapiens	103	34376.8	99.996
1837	enolase 3 (beta, muscle)	gi|77736349	Bos taurus	154	47492.6	100
2153	Isocitrate dehydrogenase 3 (NAD+) alpha	gi|109939980	Bos taurus	74	40240.5	97.057
2198	enolase 3 (beta, muscle), isoform CRA_b	gi|119610782	Homo sapiens	310	34377	100
2205	similar to glyceraldehyde 3-phosphate dehydrogenase	gi|28189619	Bos taurus	216	15527	100
2300	malate dehydrogenase 1, NAD (soluble)	gi|77736203	Bos taurus	113	36770.2	100
2682	similar to Calpain small subunit 1 (CSS1)	gi|73947828		121	28620	100
2710	triosephosphate isomerase 1	gi|61888856	Bos taurus	558	26957	100
2724	glutathione S-transferase M2 isoform 2	gi|76613069	Bos taurus	191	21305	100
1289	phosphoglucomutase 1	gi|116004023	Bos taurus	164	61906	100
Cellular stress protein						
1146	heat shock 70kDa protein 8 isoform 1	gi|5729877	Homo sapiens	711	71138.4	100
1277	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	gi|78369310	Bos taurus	115	63223.7	100
2714	heat shock protein beta-1	gi|71037405	Bos taurus	365	22736.5	100
3013	heat shock protein, alpha-crystallin-related, B6	gi|115496724	Bos taurus	163	17529.1	100

Table 2. Identification of differentially expressed proteins between meat groups showed different final shear force by MALDI TOF/TOF mass spectrometer.

Spot no	Protein Description	Accession number	Species	MOWSE	M.Wt.	Protein score C.I%
Metabolic enzyme						
1238	succinate dehydrogenase complex subunit A	gi|89574195	Bos taurus	70	62012.6	93.561
1632	mitochondrial aldehyde dehydrogenase 2	gi|115496214	Bos taurus	434	57185.2	100
1837	enolase 3 (beta, muscle)	gi|77736349	Bos taurus	154	47492.6	100
2013	Isocitrate dehydrogenase 3 (NAD+) alpha	gi|109939980	Bos taurus	73	40240.5	96.295
2179	Isocitrate dehydrogenase 3 (NAD+) alpha	gi|109939980	Bos taurus	74	40240.5	97.057
2258	GPD1 protein	gi|88682930	Bos taurus	66	37689.4	81.852
2279	L-lactate dehydrogenase B chain;Short=LDH-B	gi|118572666	-	147	37055.4	100
2303	phosphoglycerate kinase 1	gi|215983082	Ovis aries	94	45034.4	99.974
2304	fructose-1,6-bisphosphatase 2	gi|114051459	Bos taurus	463	37099.2	100
2701	triosephosphate isomerase 1	gi|61888856	Bos taurus	218	26957	100
2712	triosephosphate isomerase 1	gi|61888856	Bos taurus	448	26957	100
2816	adenylate kinase 1	gi|61888850	Bos taurus	507	21792.4	100
2819	glutathione transferase	gi|29135329	Bos taurus	132	23882.2	100
Cellular stress protein						
1135	Chain A, Crystal Structure Of Bovine Hsc70(Aa1-554)e213aD214A MUTANT	gi|78101017	-	255	61005.5	100
Miscellaneous protein						
1047	Transferrin	gi|114326282	Bos taurus	151	80388.1	100
2392	annexin VI	gi|1842109	Bos taurus	200	70178.9	100
1193	hypothetical protein isoform 2	gi|114581293	Pan troglodytes	243	77842.9	100

Fig.1 shows a representative 2DE pattern of proteins extracted from pool proteins which was collected all of tender and tough meat samples.