COMPARATIVE ANALYSIS OF PHOSPHOPROTEINS IN OVINE MUSCLE WITH DIFFERENT TENDERNESS

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Abstract – To further understand the mechanism of meat tenderization postmortem, phosphoproteins were comparatively analyzed by phosphoproteomics in ovine muscles of differnet tenderness. Forty cross-bred sheep were selected and the muscle *longissimus lumborum* were used. Muscle samples were assigned to tough and tender groups according to the shear force and myofibril fragmentation index. A combination of two-dimensional electrophoresis coupled with ProQ Diamond-SYPRO Ruby staining was used to detect phosphoproteins. Totally, 67 phosphoprotein spots were identified, among which 9 were differentially phosphorylated between the tough and tender muscles. Identification by LC-MS/MS revealed that most of the differentially phosphorylated proteins were involved in sarcomeric function or glycometabolism. In conclusion, protein phosphorylation my influence meat rigor mortis through contractile machinery and glycolysis which in turn affect meat tenderness.

Key Words – sheep, tenderization, phosphoproteomics.

I. INTRODUCTION

Tenderness is considered to be the most important quality attribute of meat. Studies show that the most common cause of unacceptability in beef, pork and lamb is toughness [1]. Post-translational modifications are key modulators of protein structure and function. Phosphorylation of proteins, a common post-translational modification, plays an important regulatory role in muscle contraction, enzyme activity, protein stability and degradation, which are all related to postmortem changes in muscle and meat quality formation. Thus, we hypothesize that protein phosphorylation/dephosphorylation regulates postmortem proteolysis and meat tendrization. Here the phosphorylation of proteins was comparatively studied between ovine muscles of different tenderness. Nine proteins were identified to be differentially phosphorylated between muscles, which were mostly involved in sarcomeric function and energy metabolism. This study provides new insight into the mechanism of meat tenderness development.

II. MATERIALS AND METHODS

Animals and sample collections

Forty 6-month old, male (not castrated) sheep (Fat-tailed sheep × tail sheep) with an average live weight of 20.08 kg (standard deviation 1.71) were slaughtered in a commercial slaughter house (Jinlong Breeding Park in the city of Shuozhou, Shanxi province, China). The *longissimus lumborum* muscle at the 13th rib was collected at 24 h postmortem. Muscle samples used for phosphoprotein analysis were immediately snap-frozen in liquid nitrogen and stored at -80 °C until analysis. Samples used for Warner Bratzler shear force (WBSF) and myofibril degradation measurements were stored at -20 °C.

Warner Bratzler shear force

The WBSF was measured as D'alessandro et al. [2]. Muscle samples were cooked in 80 °C water bath until core temperature reached 70 °C, cooled at 4 °C for 6 h and then cut into 1 × 1 × 3 cm in size with the long axis paralleled to the muscle fibers direction. The WBSF values were measured using a TA-XT2i texture analyzer (Stable Micro System Ltd., Godalming, UK) equipped with a WBSF device.

Myofibril degradation

Myofibril fragmentation index (MFI) was measured as described by Culler et al. [3].

Grouping
The muslce samples were assigned to tough and tender groups according to the WBSF data. The MFI was measured as a checking parameter for the WBSF result. Four samples were selected for both groups.

Myofibril ultra-structure
Muscle ultra-structure was examined by transmission electron microscopy as described by Mestre Prates et al. [4].

Proteins extraction
Muscle proteins were extracted as previously described by Lametsch et al. [5].

Two dimensional electrophoresis (2-DE)
The 2-DE was performed as described by Lametsch et al. [5]. Equal amount of total protein was used during 2-DE procedures. After electrophoresis, gels were stained with Pro-Q Diamond (Invitrogen, USA) according to the manufacturer's instruction to identify phosphorylated proteins and imaged using Typhoon Triovariable mode imager system (GE Healthcare, USA). Gels were then re-stained with Coomassie blue to visualize total proteins and imaged again. The 2-DE gel images were analyzed using the Image Master Platinum 5 software package to identify and quantify the protein spots.

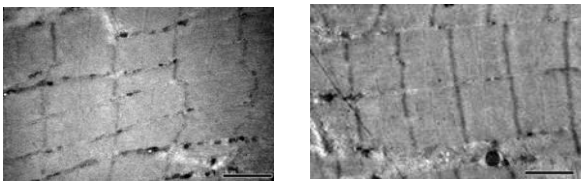
Protein identification
Protein spots were excised from gels and subjected to in-gel trypsin digestion. Proteins were identified by LC-MS/MS (Thermo Q-Exactive, Thermo Scientific, Massachusetts, USA).

Statistical analysis
The data were analyzed using SPSS (v17.0, SPSS Inc., Chicago, USA). The statistical analyses were performed using independent-samples T test. Reported p values were evaluated at a 5% significance level. Date were expressed as means ± S.D..

III. RESULTS AND DISCUSSION

Meat tenderness evaluation
Meat samples in tender group were characterized by lower WBSF (75.75 ± 7.39 vs. 130.64 ± 24.25 N, $p < 0.01$), higher MFI (99.55 ± 4.31 vs. 73.40 ± 3.20, $p < 0.01$) and longer sarcomere length (1.15 ± 0.06 vs. 0.94 ± 0.08 μm, $p < 0.01$, Fig. 1) in comparison with tough group.

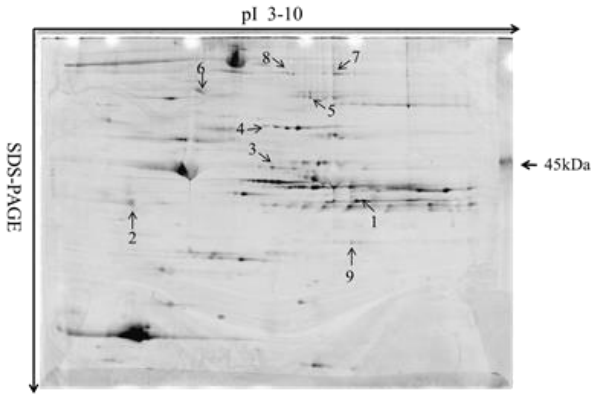
Figure 1. The ultra-structure of muscles with different tenderness (magnification: 30000×).



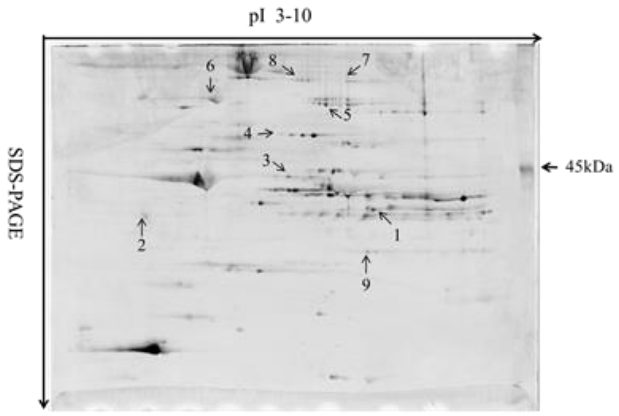
Tender group Tough group

Comprehensive phosphoproteome expression profiling
The 2-DE gels stained with Pro-Q Diamond were presented in Fig. 2. Totally, 67 phosphoprotein spots were identified, among which 9 differentially phosphorylated protein spots were found (Table 1). The isoelectric points (pI) of these proteins were 5-8 and the molecular weights were 30-130 kDa. The phosphorylation abundance of NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (NDUFS2), myosin binding protein H (MYBPH), glycogen phosphorylase (GP), alpha-actinin-3(ACT3) and myosin-binding protein C 2 (MYBPC2) in tender group was higher than that

Figure 2. Annotation of the phosphoproteins in groups with different tenderness.



Tough group



Tender group

Figure 3. Abundance of the different phosphoproteins in groups with different tenderness.

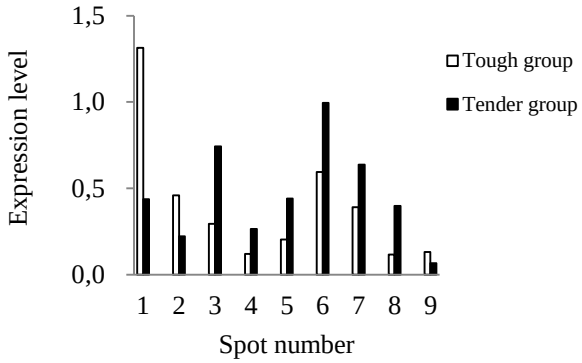


Table 1 Mass spectrometry information of the differentially phosphorylated proteins

Spot No.	Accession No.	Protein names	MW (Da)	pI	Score	Matched peptides	Coverage of sequence (%)
1	D7R7V6	GAPDH	36110	8.51	79	4	3
2	B2LU28	TPM1	32732	4.69	1692	44	44
3	P17694	NDUFS2	52921	6.51	282	11	22
4	Q0VBZ1	MYBPH	53647	5.71	253	7	4
5	O18751	GP	97702	6.65	4922	182	67
6	Q0III9	ACT3	103713	5.31	1457	53	55
7	E1BNV1	MYBPC2	127847	6.45	2132	71	31
8	E1BNV1	MYBPC2	127847	6.45	985	38	16
9	Q3SZX4	CA3	29637	7.71	809	28	30

in tough group. The phosphorylation abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tropomyosin and carbonic anhydrase 3 (CA3) in tender group was lower than that in tough group (Fig. 3).

Differentially phosphoprylated proteins between tender and tough groups

The enzyme GAPDH plays a central role in carbohydrate metabolism. It converts D-glyceraldehyde-3-phosphate and NAD^+ to 1, 3-bisphosphoglycerate and NADH in the glycolytic pathway or the reverse reaction in the gluconeogenic pathway. A study showed that activation of several serine/threonine protein kinases and phosphorylation of GAPDH significantly decreased GAPDH activity [6]. This suggests that the phosphorylation state of GAPDH does play a significant role in regulating its activity. Serine/threonine site phosphorylation is possible to decrease the rate of glycolysis.

The end-to-end interactions of adjacent tropomyosin (TPM) molecules were strengthened when tropomyosin is phosphorylated. Phosphorylation is thus essential for long range cooperative activation along the thin filament [7]. The protein phosphorylation level of tropomyosin in the tough group was higher than that in the tender group, so the long range cooperative activation along the thin filament should be more strengthened in tough group. The higher expression level of phosphorylated tropomyosin in tough group may

play an important role in making the muscle of this group less tender than the other group.

Phosphorylase kinase can phosphorylate glycogen phosphorylase b on serine 14, change its structure and transform it into the active form [8, 9]. The glycolysis process and pH decline in postmortem muscle were accelerated consequently and meat tenderness was influenced.

The abundance of phosphorylated ACT3 in tender group was higher than in tough group. The complex of α-actinin-actin plays an important role in maintaining cell shape. Once the ACT bound to actin filaments were phosphorylated, they disassociated from actin filaments [10]. Therefore, the phosphorylation of ACT causes the complex dissolute and it may be a reason for better tenderness in tender group.

The abundance of phosphorylated myosin-binding protein C in tender group was higher than tough group. Phosphorylation of myosin-binding protein C reduces the binding affinity of N-terminal domains of myosin-binding protein C for actin, suggesting that myosin-binding protein C phosphorylation may act to reduce drag on cross-bridge cycling and accelerate cross-bridge detachment [11]. The tenderization process is speed up as a result.

IV. CONCLUSION

Most of the differentially phosphorylated proteins were with sarcomeric function or involved in glycometabolism. Protein phosphorylation may influence meat rigor mortis through contractile machinery and glycolysis, which in turn affect meat tenderness.

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