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Quantitative phosphoproteomic analysis of ovine muscle with different postmortem glycolytic rates

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ABSTRACT

Phosphorylation regulates protein structure, function, cell signaling and enzyme activities within cells. Postmortem changes of muscle to meat are partially determined by the structure, function and enzyme activities of proteins. To further understand the mechanisms regulating postmortem changes, ovine muscles with different glycolytic rates were subjected to quantitative phosphoproteomic analysis. Totally 116 unique phosphopeptides matched to 99 phosphoproteins were detected to be different in abundance among the fast, moderate and slow glycolytic rate muscles. Of which, 24 phosphoproteins clustered into glycolysis and muscle contraction were selected after bioinformatics analysis. Quantitative analysis showed that phosphorylation of pyruvate kinase, phosphoglucomutase 1, enolase and fructose-bisphosphate aldolase was correlated with glycolytic rate early postmortem. In addition, some myofibrillar proteins were detected to be differentially phosphorylated. In summary, this study revealed that protein phosphorylation at early postmortem may indirectly affect the glycolysis pathway through the regulation of proteins involved in glycolysis and muscle contraction.

1. Introduction

The physical and chemical changes occurring in muscle after slaughter contribute to the postmortem development of meat quality or the maturation of meat (Paredi, Raboni, Bendixen, de Almeida, & Mozzarelli, 2012). Among these changes, glycolysis is probably most important to final meat quality formation as it influences the temperature of carcasses, the progress of cell apoptosis and rigor mortis, the pH of muscle and proteolysis postmortem which in turn, directly or indirectly, regulate meat quality traits, like meat color, water holding capacity, tenderness and so on (Chaudhry & Bhimji, 2018; Honikel, 2014). Fast and excessive glycolysis and pH declines postmortem cause inferior meat, such as pale, soft and exudative (PSE) meat and acid meat. PSE meat is a big problem to meat industry, which brings about an economic loss of millions of dollars annually in America (Li & Wick, 2001). Currently, it is believed that the fast glycolysis and thus pH decline early postmortem is the cause of PSE meat (Immonen & Puolanne, 2000; Shen et al., 2006). In fact, many literatures have reported that pH decline early postmortem is critical to meat quality development (Huang et al., 2011; Lindahl, Henckel, Karlsson, & Andersen, 2006). For these reasons, it is necessary to understand the mechanism regulating glycolysis in postmortem muscle, which has been being studied for decades, but not fully understood.

Reversible protein phosphorylation, a pervasive post translational modification, plays a regulatory role in protein structure, function, cell signaling and enzyme activity regulation. Phosphorylation usually occurs at serine, threonine and tyrosine residues catalyzed by upstream protein kinase through the addition of a covalently bound phosphate group. Most glycolytic enzymes are phosphoproteins, including the three rate-limiting enzymes, hexokinase, phosphofructokinase and pyruvate kinase. Phosphofructokinase is activated after phosphorylation by AMP-activated protein kinase (AMPK) to up-regulate glycolysis and maintain intracellular ATP homeostasis in ischemic heart (Marsin et al., 2000). Pak1-mediated phosphorylation of phosphoglycerate mutase at threonine 466 significantly increases the enzyme activity (Gururaj, Barnes, Vadlamudi, & Kumar, 2004). Consistently,

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glyceraldehyde-3-phosphate dehydrogenase purified from skeletal muscle of hibernating mammals shows lower phosphorylation level compared to control (Bell & Storey, 2014). However, these studies are not carried out under postmortem conditions, difference may exist in the machinery regulating glycolysis antemortem and postmortem. In addition, protein phosphorylation regulate rigor mortis via its action on glycolysis and muscle contraction (Li Chen, Li, Li, Chen, Everaert, & Zhang, 2018; Huang, Larsen, Palmisano, Dai, & Lametsch, 2014). The proteolytic susceptibility of myofibrillar proteins to degradation by μ-calpain has been reported to be prevented by phosphorylation, and the degradation of some myofibrillar proteins is enhanced by dephosphorylation (Li, Li, Du, Shen, & Zhang, 2018; Li et al., 2017). All these studies show that protein phosphorylation plays a wide-range effect on the postmortem conversion of muscle to meat. Thus, it is necessary to have an overview of protein phosphorylation in postmortem muscle to understand its function.

To further our understanding of the mechanism regulating postmortem changes and meat quality development, especially the biochemistry of postmortem glycolysis, we profiled phosphoproteins in ovine muscle with different glycolytic rate early postmortem using high-through put quantitative proteomic tools and differential phosphorylated proteins were subjected to bioinformatics analysis. We hypothesized that protein phosphorylation plays an important role in the postmortem development of meat quality, which may be involved in the regulation of glycolysis, muscle contraction and rigor mortis and/or meat tenderization. It is anticipated to identify some key phosphoproteins critical to the conversion of muscle to meat and further our knowledge about postmortem meat quality formation.

2. Materials and methods

2.1. Sample preparation

Sixty male sheep (Small Tail Han Sheep× Mongolia sheep, carcass weight 24.0 ± 1.65 kg) were conventionally Halal slaughtered (severing the trachea, carotid arteries, and esophagus) at a local commercial abattoir according to the guidelines of Experimental Animals. The *longissimus thoracis* muscles were removed within 30 min, pH values were measured at 0.5, 2, 6, 12, 24, 48, 72 h postmortem and samples were snap frozen in liquid nitrogen. Based on muscle pH values at 0.5 h ($pH_{0.5h}$) and 6 h (pH_{6h}) postmortem, 18 sheep carcasses were chose and grouped into three (six sheep samples in each group): fast glycolytic rate (F) group ($pH_{6h} < 5.75$), moderate glycolytic rate (M) group ($5.75 < pH_{6h} < 6.20$) and slow glycolytic rate (S) group ($pH_{6h} > 6.20$). Ovine muscles collected at 0.5 h postmortem were used for proteomic analysis.

2.2. Protein extraction

Proteins for phosphoproteomic analysis were extracted and processed by the literature (Zhu et al., 2014). Two random samples in the same group were mixed as a replication (six sheep samples in each group). Muscles at 0.5 h postmortem and SDT buffer (4% SDS, 100 mM Tris-HCl, 1 mM DTT, pH 7.6) were added into a 2 mL tube with quartz sand and a 1/4 in. ceramic bead MP 6540-424, then homogenized (24 × 2, 6.0 m/s, 60 s, twice) and sonicated (100 W, 10 s, ten times). After incubating for 15 min in boiling water, the supernatant centrifuged at 14,000*g* for 10 min was filtered with 0.22 μm filters. The filtrate was divided and stored at −80 °C after determining the concentration of protein with BCA Protein Assay Kits.

2.3. Filter-aided sample preparation (FASP Digestion) and iTRAQ labeling

Proteins were digested using FASP procedures as described in literature (Li, Li, Li, Xin, Wang, Shen, et al., 2018). Digested peptides (100 μg) were labeled with iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems).

2.4. Enrichment of phosphorylated peptides

Titanium dioxide was used for phosphorpeptide enrichment as in literature (Larsen, Thingholm, Jensen, Roepstorff, & Jørgensen, 2005). The labeled peptides were reconstituted in 500 μL 1 × DHB buffer and mixed with the treated TiO_2 beads, then centrifuged at 5000*g* for 1 min after agitating for 2 h. TiO_2 beads were washed with washing buffer I (30% acetonitrile/3% trifluoroacetic acid) and washing buffer II (80% acetonitrile/0.3% trifluoroacetic acid) for 3 times, and then transferred to a new tube. After elution, collected phosphopeptides were lyophilized for further analysis.

2.5. HPLC-MS/MS analysis

Samples were separated by reverse phase trap column and the peptides fractions were analyzed by Q Exactive mass spectrometer that was coupled to Easy nLC for 240 min. The details were performed according to the literatures (Jiang et al., 2016; Li et al., 2018).

2.6. Data processing

MS/MS spectra data was analyzed with Mascot 2.2 (AnnSofi Sandberg, 2012) and Proteome Discoverer 1.4 (Thermo Electron, San Jose, CA) engine against the Uniprot database and the reversed database. The search parameters were set following the literature (Li et al., 2018).

2.7. Bioinformatic analysis

All the software and database used for bioinformatics analysis were list in the Table 1.

3. Result

3.1. pH value

Glycolysis transfers glucose into lactate under anaerobic conditions, with the drop of muscle pH values. The pH values of muscles are presented in Table 2. The pH values were decreasing within 24 h postmortem, then kept stable afterwards. Ovine muscle pH values were

Table 1
The software and database used for bioinformatics analysis.

Analysis	Software/database
Hierarchical clustering analysis	Java Treeview software (http://jtreeview.sourceforge.net) and the Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm)
Protein sequences	UniProtKB database (Release 2016_10)
GO mapping and annotation	Blast2GO (Version 3.3.5)
KEGG pathway	Kyoto Encyclopedia of Genes and Genomes database (http://geneontology.org/)
Motif	motif-X algorithm (http://motif-x.med.harvard.edu/motif-x.html)
Protein–protein interaction	IntAct molecular interaction database (http://www.ebi.ac.uk/intact/)

Table 2
The pH value in muscle of the three glycolytic rate groups.

	F group	M group	S group
0.5 h	6.333 ± 0.059^{az}	6.606 ± 0.093^{ay}	6.835 ± 0.085^{ax}
2 h	6.066 ± 0.060^{bz}	6.481 ± 0.129^{by}	6.770 ± 0.113^{ax}
6 h	5.687 ± 0.036^{cz}	5.973 ± 0.014^{cy}	6.266 ± 0.053^{bx}
12 h	5.487 ± 0.045^{d}	5.660 ± 0.071^{d}	5.798 ± 0.137^{c}
24 h	5.434 ± 0.041^{d}	5.447 ± 0.019^{e}	5.534 ± 0.154^{d}
48 h	5.469 ± 0.067^{d}	5.465 ± 0.048^{e}	5.544 ± 0.183^{d}
72 h	5.468 ± 0.064^{d}	5.403 ± 0.025^{e}	5.522 ± 0.154^{d}

Different letters (x, y, z) at the same row are significant difference in the three groups ($P < 0.05$). Different letters (a–e) at the same column are significant difference at different postmortem time ($P < 0.05$).

significantly different at 0.5, 2 and 6 h postmortem among the three groups, with S group being higher than M group and M group being higher than F group. Muscles in S group had the highest pH values, while the muscles in F group had the lowest pH value.

3.2. Phosphoprotein identification and motif analysis

A total of 1905 phosphopeptides were identified, which were assigned to 704 phosphoproteins. Ninety-seven phosphopeptides were determined to be different in abundance between F and M groups, of which 67 peptides were down regulated and 30 were up regulated compared to M group. Forty-one phosphopeptides were significant difference in abundance between M and S groups, of which 15 peptides were down regulated and 26 were up regulated compared to S group. Eighty-nine phosphopeptides were differently expressed in F and S groups, of which 51 peptides were down regulated and 38 were up regulated compared to S group. A total of 116 unique phosphopeptides (Supplementary material 1), matching to 98 phosphoproteins and containing 188 phosphorylation sites, were significant difference in abundance among the three groups after one way ANOVA. Generally, 160 phosphoserine, 26 phosphothreonine and 2 phosphotyrosine residues (ratios of 85.11%, 13.83% and 1.06% respectively) were identified. Sixteen putative phosphorylation motifs were identified after analyzing with the Motif-X software, 13 serine motifs PS and 3 threonine motifs PT were included (Supplementary material 2). It is logical to deduce that proteins phosphorylated at these sites may play a role in glycolysis pathway.

3.3. Hierarchical clustering analysis

The 116 phosphopeptides with significant difference in phosphorylation level (listed in Supplementary material 1) were subjected to hierarchical clustering analysis in Fig. 1. All 116 phosphopeptides were shown in 116 rows. Different colors in the same row represent phosphorylation levels of one phosphopeptide among the three groups. Red color represents a high abundance and a high phosphorylation level while the green color means a low abundance and a low phosphorylation level. Phosphorylation levels of all the 116 phosphopeptides were significant difference among three groups, especially between the F and S groups. The phosphopeptides with high phosphorylation level in the F group (upper part in Fig. 1) has a low phosphorylation level in the S group, while the phosphopeptides with low phosphorylation level in the F group (lower part of Fig. 1) has a high phosphorylation level in the S group. At the bottom, a few phosphopeptides expressed irregularly among the three groups. Hierarchical cluster analysis visualizes the experimental specificity and reproducibility.

3.4. Functional enrichment analysis

GO terms enrichment and KEGG pathway enrichment were performed to obtain the important information about the regulation

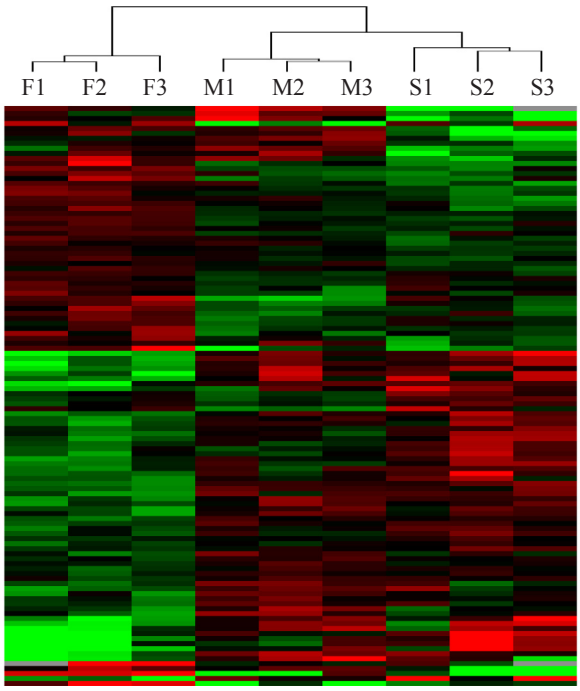

Fig. 1. Hierarchical clustering analysis among the three glycolytic rate groups. Note: Muscle samples are displayed in columns and classified by phosphoproteomic subtypes as indicated by different glycolytic rate. Two random samples in the same group were mixed as a replication. F1, F2 and F3 are the three replications in fast glycolytic rate group. M1, M2 and M3 are the three replications in moderate glycolytic rate group. S1, S2 and S3 are the three replications in slow glycolytic rate group. The same row represents one phosphopeptide, and different colors represent phosphorylation levels. A redder color means a higher phosphorylation level and a greener color means a lower phosphorylation level of the phosphopeptide.

mechanism of glycolysis (Fig. 2). Most of the phosphoproteins expressed differentially in three groups after GO terms enrichment are involved in the regulation of phosphoprotein phosphatase activity, regulation of protein dephosphorylation, relaxation of muscle, regulation of phosphatase activity, glycolytic process through fructose-6-phosphate, canonical glycolysis, glucose catabolic process to pyruvate, NADH regeneration, glycolytic process through glucose-6-phosphate, muscle contraction, skeletal muscle tissue regeneration, glucose catabolic process. All these biological processes are focused on glycolysis and muscle contraction. The phosphoproteins belonged to these biological processes may be glycolytic rate related and play a role in glycolysis. In addition, 24 glycolytic rate related phosphoproteins were identified in the present study (Table 3).

The primary four pathways were determined after KEGG pathway enrichment, including RNA degradation, herpes simplex infection, antigen processing and presentation, and spliceosome. All the 24 phosphoproteins mainly focus on the following glycolysis maps after annotation which were Pyruvate metabolism, Pentose phosphate pathway, Tight junction, Calcium signaling pathway and Regulation of actin cytoskeleton.

3.5. Protein-protein interaction network analysis

All the differentially expressed phosphoproteins were used to perform protein–protein interaction network analysis and three different clusters were showed in Fig. 3. The largest one is the muscle contraction related proteins, and the closest interaction one is the glycolytic enzymes. Thirteen proteins were identified as the muscle contraction related proteins, 6 proteins were identified as glycolytic enzymes and 5 other proteins were identified in the present study.

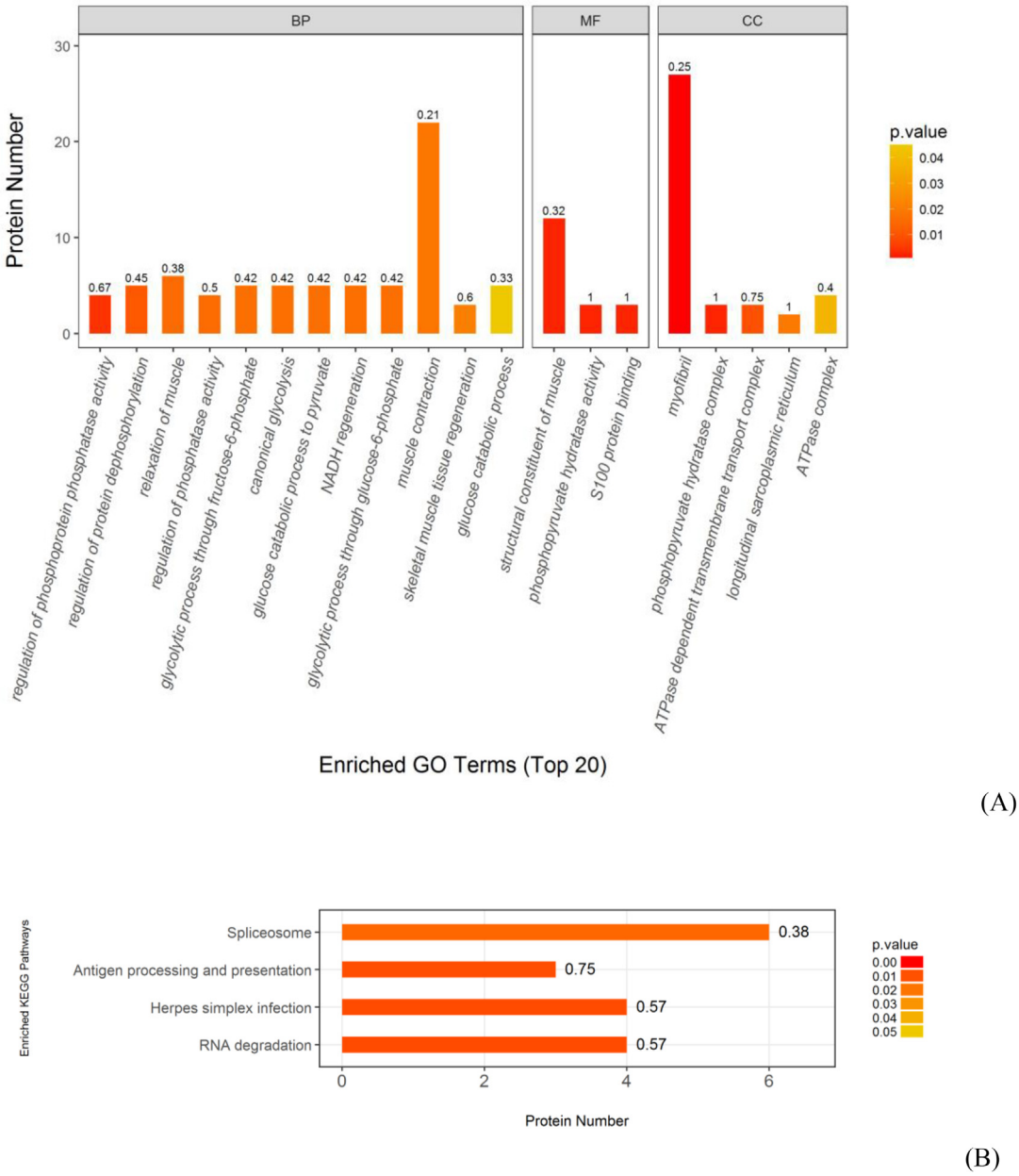

Fig. 2. GO terms enrichment and KEGG pathway enrichment of differently phosphorylated proteins in muscles of different glycolytic rate groups. (A) The enriched GO terms of differently expressed phosphoprotein among the three groups. (B) The enriched KEGG pathways of differently expressed phosphoprotein among the three groups. Note: The abscissa in the figure A indicates the enriched GO function, BP, MF and CC represent biological process, molecular function and cellular component respectively. The ordinate in the figure B indicates the significant KEGG pathway. The numbers above the bars called richfactor, it indicates the ratio of proteins corresponding to the phosphopeptides significantly different in phosphorylation level to all identified proteins.

3.6. Quantitative analysis of phosphopeptides

More than one phosphopeptide and phosphosite were identified in the 24 glycolytic rate related phosphoproteins in the present study. Totally, 390 phosphopeptides were identified from 24 phosphoproteins, in which 32 phosphopeptides were different in phosphorylation level among the three groups (Table 3). Moreover, the phosphopeptides and phosphosites revealed different phosphorylation level among the three groups.

3.6.1. Glycolytic enzymes

Ten phosphopeptides were identified from pyruvate kinase, but only phosphorylation at Thr155 was up regulated in S group and down regulated in F group ($P < 0.05$). Ten phosphopeptides were identified from phosphoglucomutase 1, but only phosphorylation at Ser402 was up regulated in S group and down regulated in F group ($P < 0.05$). Four phosphopeptides were identified from enolase 2, but only phosphorylation at Ser177 was up regulated in M and S groups and down regulated in F group ($P < 0.05$). Eight phosphopeptides were identified from enolase 3, but only phosphorylation at Ser176 was up regulated in M and S groups and down regulated in F group ($P < 0.05$). Three phosphopeptides were identified from fructose-bisphosphate aldolase, but only phosphorylation at Ser124 and Ser127 was up regulated in M and S groups and down regulated in F group ($P < 0.05$).

3.6.2. Muscle contraction

Sixty-five phosphopeptides were identified from myosin heavy chain 2, but only phosphorylation at Thr422 was up regulated in M and

Table 3
The 32 phosphopeptides and their phosphosites of 24 glycolytic rate related phosphoproteins.

No	Accessions	Phosphoproteins	Sequence (significantly different among groups)	Phosphosite in peptide	Location in phosphoproteins	F	M	S
1	W5NQP9	Fructose-bisphosphate aldolase	gILAADEsVGsMAk	S(8): 100.0; S(11): 100.0	S124,S127			
2	W5QC41	Pyruvate kinase	gPEIRtGLIk	T(6): 100.0	T155			
3	W5PJB6	Phosphoglucomutase 1	fFGNLMDAsk	S(9): 100.0	S402			
4	W5P5C0	Enolase 2	lAMQEFmILPVGAEsFR	S(15): 100.0	S177			
5	W5P663	Enolase 3	lAMQEFmILPVGAsSFR	S(14): 97.2; S(15): 2.8	S176			
6	W5PT09	Myosin heavy chain 2	gQTVEQVtNAVGALAk	T(3): 0.0; T(8): 100.0	T422			
7	W5Q0I1	Myosin binding protein C	sGEGQDDAGELDFSGLLk	S(1): 100.0; S(14): 0.0	S151			
			fDScSFDLEVHESTGttPNIDIR	S(3): 0.0; S(5): 0.0; S(13): 13.3; T(14): 13.3; T(16): 86.7; T(17): 86.7	T138,T139			
8	W5Q754	Titin	tSEMEASSsVR	T(1): 0.0; S(2): 0.0; S(7): 2.2; S(8): 2.2; S(9): 95.6	S34313			
			aEFVcsISk	S(6): 97.4; S(8): 2.6	S14375			
			rsLGDIsDEELLLPIDDYLAmk	S(2): 100.0; S(7): 100.0; Y(18): 0.0	S33892,S33897			
			vTsDNLmSR	T(2): 2.3; S(3): 95.4; S(8): 2.3	S18570			
			tIVsTAQISETR	T(1): 0.0; S(4): 2.1; T(5): 95.8; S(9): 2.1; T(11): 0.0	T210			
9	W5PFV9	Nebulin	ftSVTDsLEQVLAk	T(2): 97.4; S(3): 2.5; T(5): 0.1; S(7): 99.9	T2938, S2943			
			mVGFQsLQDDPk	S(6): 100.0	S1099			
10	W5Q1Q5	Myotilin	sRssSRGDmSDQDAIQEk	S(1): 97.7; S(3): 67.3; S(4): 67.3; S(5): 67.3; S(10): 0.6	S230, S232, S233, S234			
11	B2LU28	TPM1	aIsEELDHALNDMTsI	S(3): 100.0; T(14): 50.0; S(15): 50.0	S271, T282/S283			
			lEksIDDLEDELYAQk	S(4): 100.0; Y(13): 0.0	S252			
12	W5QFE3	Cardiac phospholamban	astIEmPQQAR	S(2): 100.0; T(3): 100.0	S16, T17			
13	W5NU05	Myosin light chain kinase 2	iSsSGALMALGV	S(2): 1.3; S(3): 97.4; S(4): 1.3	S617			
14	B6VCA9	Myosin light chain 2	rAEGANsNVFSmFEQTQIQEFk	S(6): 100.0; S(10): 100.0; T(15): 100.0	S15,S19,T24			
15	A9P323	Heat shock protein 90 alpha	lGIHEDsQNR	S(7): 100.0	S443			
16	W5PDJ3	PDZ and LIM domain 3	lWsPQVTEDGk	S(3): 100.0; T(7): 0.0	S89			
17	W5PBB8	Phosphodiesterase	eWYQStIPQSPsPAPDDQEEGR	Y(3): 0.0; S(5): 0.0; T(6): 0.0; S(10): 100.0; S(12): 100.0	S585, S587			

Note: Location in peptide indicates phosphosite in peptide derived in UniProt database. F, M and S represent three groups. The color bar represents the phosphorylation level of phosphopeptide from Hierarchical clustering analysis. The phosphorylation levels were marked from red to green in abundance from high to low. A redder color means a higher phosphorylation level and a greener color means a lower phosphorylation level of the phosphopeptides. "–" represents there is no significantly different phosphopeptide in the phosphoprotein.

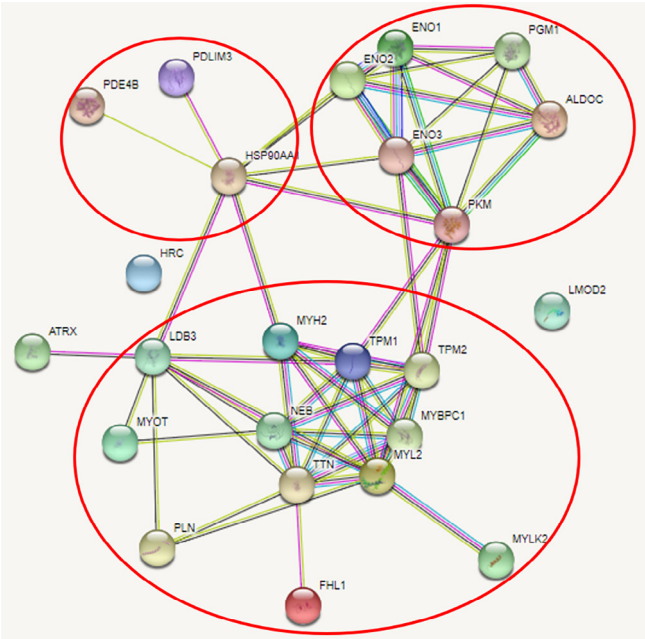

Fig. 3. Protein–protein interaction networks of identified glycolytic rate related phosphoproteins in ovine muscle. Different clusters of interacting proteins were identified using STRING to obtain a high confidence evidence network.

S groups and down regulated in F group ($P < 0.05$). Four phosphopeptides were identified from myosin light chain kinase 2, but only phosphorylation at Ser617 was up regulated in F group and down regulated in M and S groups ($P < 0.05$). Six phosphopeptides were identified from myosin light chain 2, but only phosphorylation at Ser15 was up regulated in M group and down regulated in F group ($P < 0.05$). Three phosphopeptides were identified from myosin binding protein C, the phosphorylation at Ser151 was up regulated in M and S groups and down regulated in F group ($P < 0.05$), the phosphorylation at Thr138 and Thr139 was up regulated in M group and down regulated in F group ($P < 0.05$). One hundred and thirty-three phosphopeptides were identified from titin, while 5 of them were different among the three groups. The phosphorylation at Ser34313, Ser18570 and Thr210 was up regulated in M and S groups and down regulated in F group ($P < 0.05$), the phosphorylation at Ser14375 was up regulated in M group and down regulated in F group ($P < 0.05$), the phosphorylation at Ser33892 and Ser33897 was up regulated in M group and down regulated in S group ($P < 0.05$). Twenty-eight phosphopeptides were identified from nebulin, of which the phosphorylation at Ser2943 and Thr2938 was up regulated in M and S groups and down regulated in F group ($P < 0.05$), the phosphorylation at Ser1099 was up regulated in S group and down regulated in F group ($P < 0.05$). Eight phosphopeptides were identified from myotilin, but only phosphorylation at Ser230, Ser232, Ser233 and Ser234 was up regulated in M and S groups and down regulated in F group ($P < 0.05$). Twelve phosphopeptides were identified from tropomyosin 1, but only phosphorylation at Ser271 was up regulated in F group and down regulated in M group ($P < 0.05$). Five phosphopeptides were identified from

cardiac phospholamban, but only phosphorylation at Ser16 and Thr17 was up regulated in M and the S groups and down regulated in F group ($P < 0.05$).

4. Discussion

4.1. Phosphorylation of glycolytic enzymes and glycolysis

Glycolysis is a sequence of enzymatic reactions which are determined by the activities of glycolytic enzymes. Several studies revealed that the glycolytic enzymes influenced the transition process of muscle to meat for its activities changed after slaughter (Huang et al., 2011; Werner, Natter, & Wicke, 2010). Protein phosphorylation plays a regulatory role in protein structure, function, signaling and activity regulation. Most glycolytic enzymes have been reported to be phosphorylated and phosphorylation increase enzymes' activity or stability (Reiss, Kanety, & Schlessinger, 1986; Sale, White, & Kahn, 1987). Pyruvate kinase, phosphoglucomutase 1, enolase2, enolase3 and fructose-bisphosphate aldolase were identified to be the glycolytic rate related phosphoproteins in the present study, which were detected to be phosphorylated at Thr155, Ser402, Ser177, Ser176, S124 and Ser127, respectively. Their phosphorylation levels were significantly different among the three groups, with phosphorylation levels being significantly higher in M and S groups than in F group (Table 3).

Pyruvate kinase, a rate-limited glycolytic enzyme, catalyzes the conversion of phosphoenolpyruvate to pyruvate irreversibly. Phosphorylation at Thr155 shows significantly different among the three groups, with phosphorylation levels being higher in S group and lower in F group. The trends is consistent with Chen's result (Li Chen, Li, Li, Chen, Everaert, & Zhang, 2018). It has been reported that pyruvate kinase has two isoforms in normal muscle. Isoforms 2 arose from isoform 1 through phosphorylation. Phosphorylation of pyruvate kinase could result in an additional, more acid stable enzyme isoform, and maintain high activity in PSE meat (Zhang & Liu, 2017). Therefore, the low phosphorylation level in F group at early postmortem indicates the high activity of pyruvate kinase.

The activity of phosphoglucomutase 1 may alter after phosphorylation, that can stimulate the drop of pH value (Gururaj et al., 2004). Fructose 1,6-bisphosphate aldolase is one of the phosphoproteins that may be directly linked to postmortem pH decline (D'Alessandro & Zolla, 2013). The activity of enolase 1 was reduced at hyper-phosphorylation condition (Jin et al., 2008). Based on these reports, we supposed that phosphorylation levels of these glycolytic enzymes were related to glycolytic rate at early postmortem.

Glycolysis is a dynamic physiological and developmental processes. In glycolysis pathway, the metabolism of one molecule of glucose to two molecules of pyruvate has a net yield of two molecules of ATP, with concomitant production of lactate and muscle pH decline. Protein phosphorylation was regulated by protein kinase which transfers a phosphate group from a nucleoside triphosphate (usually ATP) and covalently attaches it to amino acids. Phosphorylation of pyruvate kinase could maintain high activity in low pH value. Therefore, ATP and low pH will also affect phosphorylation.

4.2. Phosphorylation of muscle contraction related proteins and glycolysis

The phosphorylation of sarcoplasmic and myofibrillar proteins would doubtless alter with the exhaustion of ATP and the release of Ca^{2+} in postmortem muscle. Myosin heavy chain 2, titin, myosin binding protein C, myosin light chain 2, tropomyosin, myosin light chain kinase 2 and so on clustered in muscle contraction were significantly different in phosphorylation levels among the three muscle groups. Some of them such as tropomyosin, myosin heavy chain 2, myosin light chain 2 have been reported to be differentially phosphorylated in postmortem pig samples (Huang, Larsen, & Lametsch, 2012). The change in phosphorylation levels of muscle contraction related proteins would affect the progress of rigor mortis development. Phosphorylation of phosphofructokinase regulates its kinetic activity and binding affinity with F-actin. Stimulation of muscle contraction increases its phosphorylation and binding to the F-actin (Luther & Lee, 1986). Tropomyosin phosphorylation is related to the functional plasticity of the thin filaments (Heeley, Watson, Mak, Dubord, & Smillie, 1989). The increased phosphorylation of tropomyosin and myosin heavy chain 2 might be a response to the stretch of muscle (Huang et al., 2014). The phosphorylation of myosin light chain 2 is associated with muscle contraction, and the extent of constriction is positively correlated with the phosphorylation of myosin light chain 2 (Chen et al., 2016). Gao's results suggest that the phosphorylation of myosin light chain enhances actomyosin dissociation and has a negative influence on actomyosin ATPase activity (Gao, Li, Li, Du, & Zhang, 2017). The activity of ATPase in turn influence the phosphorylation level of sarcoplasmic and myofibrillar proteins.

4.3. Glycolysis regulation pathway through phosphorylation

4.3.1. Enzyme activities regulation pathway

Protein phosphorylation regulates most of the important processes in muscle, such as metabolism and contraction (Hou et al., 2010; Lundby et al., 2012). A considerable number of studies has revealed that many enzymes and myofibrillar proteins are regulated by phosphorylation through the change of activities in postmortem muscle (Muroya et al., 2007; Schwägele, Buesa, & Honikel, 1996; Shen & Du, 2005). Glycogen phosphorylase exists in phosphorylated glycogen phosphorylase a and dephosphorylated glycogen phosphorylase b forms. Glycogen phosphorylase b, after phosphorylation at serine 14, transforms to glycogen phosphorylase, which was more activated, thus accelerating the glycolysis process (Aizawa et al., 2017). It has been reported that the activation of GAPDH was up-regulated when the phosphorylation of GAPDH is increased (Baba et al., 2010). The activity of pyruvate kinase is inhibited when it is phosphorylated at Serine 215. Based on these previous studies, we supposed that protein phosphorylation may alter the activity of glycolytic enzymes and the rate of glycolysis.

4.3.2. μ-calpain degradation pathway

The disruption of myofibril structure by proteolytic enzymes and degradation of myofibrillar proteins are two mechanisms for meat tenderization, among which μ-calpain are considered to be primary protease for protein hydrolysis during meat aging. The rate of μ-calpain autolysis is influenced by the rate of pH decline. A faster glycolysis directly result in an early appearance of the autolyzed form of the calpain (Melody et al., 2004). It was confirmed that proteolytic susceptibility of myofibrillar proteins to degradation by μ-calpain was prevented after phosphorylation (Li et al., 2017). In addition, the degradation of myofibrillar was influenced by the phosphorylation of structural proteins (D'Alessandro et al., 2012). The phosphorylation of the muscle contraction related proteins was significantly lower in F group in the present study (Table 3). We speculate that fast glycolysis results an early appearance of the autolyzed form of μ-calpain, and the low phosphorylation accelerates myofibrillar protein degradation by calpain. The phosphorylation level in S group is just the opposite.

4.3.3. Energy metabolism pathway

In glycolysis pathway, the metabolism of one molecule of glucose to two molecules of pyruvate has a net yield of two molecules of ATP. Protein phosphorylation was regulated by protein kinase which transfers a phosphate group from a nucleoside triphosphate (usually ATP) and covalently attaches it to amino acids that have a free hydroxyl group such as serine, threonine, or tyrosine (Graves & Krebs, 1999). In postmortem muscle, with the rapid degradation of ATP in F group, the phosphorylation was low for the less supplement of ATP. The low level of phosphorylation was insufficient to inhabit the glycolysis pathway,

resulting a high rate of lactate formation and a fast pH decline.

4.3.4. Protein kinase activity regulation pathway

Reversible protein phosphorylation is regulated by protein kinases and phosphatases. The optimum pH value of kinases is about 6.5 while the phosphatases is more than 8.0. The muscle pH values in the F group was significantly lower than that in M and S groups, which was further away from the optimum pH of kinases This is possibly one reason for the lower phosphorylation level of proteins in F group.

5. Conclusion

Totally 116 unique phosphopeptides matched to 99 phosphoproteins were significantly different in abundance among the three muscle groups, of which 24 phosphoproteins were clustered into glycolysis and muscle contraction after GO and KEGG enrichment. Quantitative analysis showed that phosphorylation of pyruvate kinase, phosphoglucomutase 1, enolase and fructose-bisphosphate aldolase was correlated with glycolytic rate early postmortem. In addition, some myofibrillar proteins were detected to be differentially phosphorylated in the present study, indicating that protein phosphorylation may be involved in rigor mortis and meat tenderization. Further research with different postmortem time will be beneficial to further understand the regulation of protein phosphorylation on postmortem changes and meat quality development.

Conflict of interest statement

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

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