Phosphoproteomics Analysis of Postmortem Porcine Muscle with pH Decline Rate and Time Differences

Honggang Huang[1*], Martin R Larsen[2], Anders H Karlsson[1] and René Lametsch[1]
1Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Copenhagen, Denmark
2Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark
*Correspondence: Honggang Huang, email: huanghg@life.ku.dk, phone: +4535333247

***Abstract*.** The aim of this study was to characterize the protein phosphorylation in postmortem (PM) muscle and reveal the change during meat quality development. The gel-based phosphoproteomic analysis of PM porcine muscle was performed in three pig groups with different pH decline rates from PM 1h to 24 h. The sarcoplasmic and myofibrillar fractions were analyzed using gel electrophoresis in combination with a phosphoprotein specific staining. Globally, the group with fast pH decline rate had the highest phosphorylation level at PM 1 h, but lowest at PM 24 h, whereas the group with slow pH decline rate showed the reverse case. The phosphorylation level of 12 bands in sarcoplasmic fraction and 3 bands in myofibrillar fraction were significantly affected by the synergy effects of pH and time ($p<0.05$). 72 unique proteins were identified. The phosphorylation patterns of pyruvate kinase, triosephosphate isomerase-1, tropomyosin and myosin regulatory light chain 2 showed to be related to PM muscle pH decline rate and time. Our work sheds light on the potential role of protein phosphorylation on regulation of meat quality development.

***Keywords*:** Protein phosphorylation; Postmortem porcine muscle; Meat quality development

I. INTRODUCTION

Meat quality development is highly dependent on the rate and extent of the postmortem (PM) pH decline in the muscle [1]. An increased rate of early PM glycolysis contributes to fast pH decline and results in unfavored meat quality [2].

Reversible protein phosphorylation can affect many biological processes including metabolism, transcription, signal transduction through alteration of protein conformation and/or activity [3,4]. Protein phosphorylation can regulate both glycogen metabolism [5,6] and contraction activities [7,8] in muscle. In PM muscle, phosphorylation of pyruvate kinase could result in an additional, more acid stable enzyme isoform and maintain high activity in pale, soft, and exudative (PSE) meat [9], and myosin regulatory light chain 2 (MyLC2) became doubly phosphorylated during rigor formation in bovine longissimus [10].

Studying the dynamic change of protein phosphorylation in PM meat can lead to the identification of candidate regulatory proteins, and help to understand the underlying mechanisms of meat quality development. Here, we used SDS-PAGE coupled with Pro-Q Diamond-SYPRO Ruby staining and tandem MS strategy to detect the phosphoproteins, and analyze their time and pH decline rate-dependent changes in PM porcine muscle samples.

II. MATERIALS AND METHODS

The animal samples used in this work were collected from our previous work [11]. Pigs were selected and divided into three groups (10 in each group) according to their PM pH decline rate: slow pH decline (S) group (pH 3 > 6.30), intermediate pH decline (I) group (6.00 < pH 3 <6.30) and fast pH decline (F) group (pH 3 < 6.00). Muscle samples at the 8th rib were taken at 1 h, 4.5 h, 6 h and 24 h PM and stored at -80 ºC. In our experiments, five pigs were randomly selected from each pH groups for analysis.

One gram of frozen muscle sample was minced and homogenized in the ice-cold homogenizing buffer containing 100 mM Tris pH 8.3, 10 mM DTT, the Complete protease inhibitor (Roche) and phosphatase inhibitor PhosStop (Roche). The homogenized samples were centrifuged at 25,000×g, and the supernatant (containing the sarcoplasmic proteins) was carefully collected by avoiding the fat layer. The pellet (containing the myofibrillar proteins) was dissolved in heated SDS buffer. The protein concentration was determined using the BCA assay.

Both the sarcoplasmic and myofibrillar fractions of all samples were analyzed using SDS-PAGE (Invitrogen, Denmark). The gels were firstly stained with Pro-Q Diamond staining (Invitrogen, Denmark) for detection of the phosphoproteins, and followed with SYPRO Ruby (Invitrogen, Denmark) and colloidal Coomassie Brilliant Blue R-250 staining for total proteins. Images from both Pro-Q Diamond and SYPRO Ruby staining methods were analyzed with TotalLab TL120 software package (TotalLab, Germany) to compare band variances.

Protein bands were excised and subjected to in-gel trypsin digestion and protein identification using a MALDI-TOF-TOF-MS/MS instrument (4800 Proteomics analyzer, Applied Biosystems, Foster City, CA). The obtained data were searched against Swiss Prot and NCBInr database by using MASCOT search engine.

The protein phosphorylation level was ratiometricly quantified by determining the ratio of the intensity of phosphoprotein (P) in Pro-Q Diamond image to its intensity of total protein (T) in SYPRO Ruby image (P/T ratio). The effects of pH decline rate and PM time on the protein phosphorylation level were statistically analyzed using a linear mixed model.

III. RESULTS AND DISCUSSION

Apparent differences could be observed by comparison of the Pro-Q Diamond and SYPRO Ruby images (Fig.1). The phosphoproteins were clearly stained in Pro-Q Diamond image, which indicated the specificity of Pro-Q Diamond staining for phosphoproteins.

Fig.2 demonstrated that the global P/T ratio change patterns reflected remarkable differences in both sarcoplasmic and myofibrillar fractions. Globally, at PM 1 h, the phosphorylation level in F group was higher than the other two groups; however, at PM 24 h, the phosphorylation level in the F group was the lowest. From PM 1 h to 24 h, I and S groups showed similar change patterns, increased with time, but the F group showed a reverse case. Our result indicated that the muscle global phosphorylation level showed reverse change patterns between groups with different pH decline rates.

The effects of pH and time on the P/T ratio of all 19 bands were evaluated by association analysis. The result was presented in Table 1. For the sarcoplasmic fraction, P/T ratios of 12 bands were significantly affected by the synergy effects of pH and time ($p<0.05$), and P/T ratios of 4 bands were only influenced by different time points at a significant level ($p<0.05$). For the myofibrillar fraction, the P/T ratios of two bands were significantly affected by the synergy effects of pH and time ($p<0.05$), and seven bands were only significantly influenced by PM time ($p<0.05$).

All the 23 bands from sarcoplasmic fraction and 19 bands from myofibrillar fraction were excised and identified by MALDI-TOF-TOF-MS/MS. A total of 37 non-redundant proteins were identified from the sarcoplasmic fraction, and 35 non-redundant proteins were identified from myofibrillar fraction. Among the 37 identified sarcoplasmic proteins, 17 were involved in the glycogen and glycolysis metabolism, and most of them were localized in the bands where P/T ratios showed significant differences between different pH groups. The majority (22) of the identified myofibrillar proteins were putative myofibrillar proteins involved in sarcomeric functions.

Bands 7, 11, 15 and 20 were the intensively stained bands in the ProQ diamond image of sarcoplasmic sample, after MS/MS identification, glycogen phosphorylase (GP), pyruvate kinase (PK), fructose-bisphosphate aldolase C-A (ALDOA) and triosephosphate isomerase 1 (TPI1) were found to be the most abundant proteins identified from bands 7, 11, 15 and 20, respectively. For the myofibrillar sample, myosin binding protein C (MYBPC2), muscle phosphofructokinase (PFK), troponin T and tropomyosin alpha-1 (TNNT3 and TPM1) and myosin regulatory light chain 2 (MyLC2) were main proteins identified from the strongly stained bands 5, 9, 13 and 19 in the ProQ diamond image. These 8 bands were all confirmed to be significantly affected by PM pH and time. All these proteins were reported to be phosphoproteins, and their phosphorylation sites were identified [12]. The change patterns of protein phosphorylation levels of

these phosphoproteins were selectively showed in Fig. 3. They all showed to be changed with PM time, the phosphorylation levels of PK, ALDOA, MYBPC2, TNNT3, TMP1 and MyLC2 in group with fast pH decline rate were higher than the other two groups, especially from PM 1 h to 6 h, but decreased at later time. However, the phosphorylation levels of GP, TPI1 and PFK in F group were much lower than the S and I groups. The protein phosphorylation change of these proteins might be related to meat quality development.

IV. CONCLUSION

Our work revealed the phosphorylation change patterns of main sarcoplasmic and myofibrillar proteins in PM meat. The majority of the identified phosphoproteins were glycogen and glycose metabolism related enzymes in sarcoplasmic fraction and contraction related proteins in myofibrillar fraction, their phosphorylation patterns were changed with PM time and varied between groups with different pH decline rates, The phosphorylation of pyruvate kinase, triosephosphate isomerase-1, tropomyosin and myosin regulatory light chain 2 showed to be related to PM muscle pH decline rate and time. Our results indicate the involvement of protein phosphorylation in regulating meat quality development. Further studies toward quantitative analysis of specific phosphoproteins and identification of phosphorylation sites would bring more detailed information for revealing their underlying mechanisms in regulating meat quality development.

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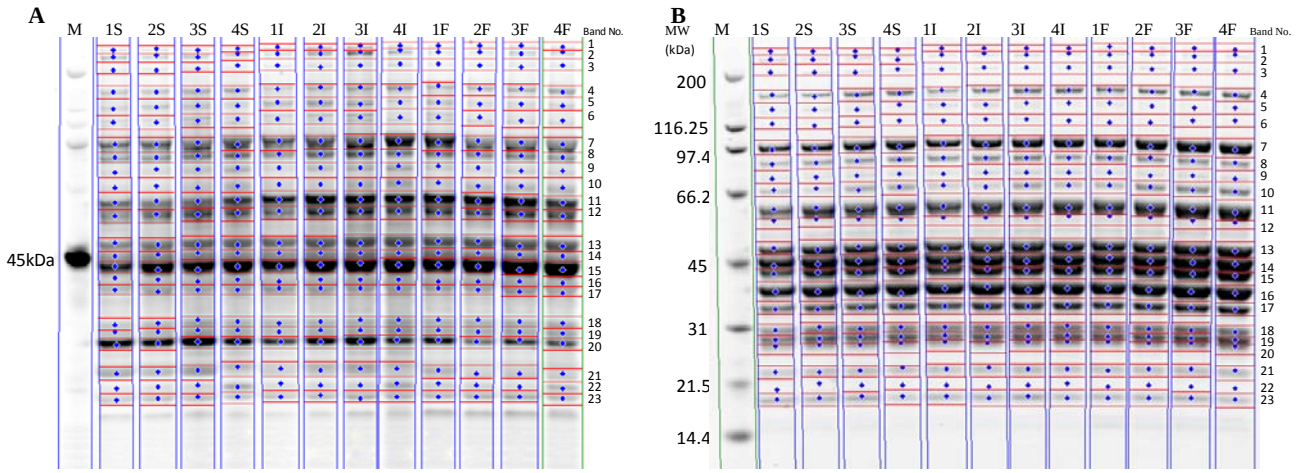

Figure 1. Gel images of phosphoproteins and total proteins from sarcoplasmic fraction. (A) Image stained with Pro-Q Diamond. (B) Image stained with SYPRO- Ruby.1, 2, 3 and 4 represented postmortem1 h, 4.5 h, 6 h and 24 h, S, I and F represented slow, Intermediate and fast pH decline groups.

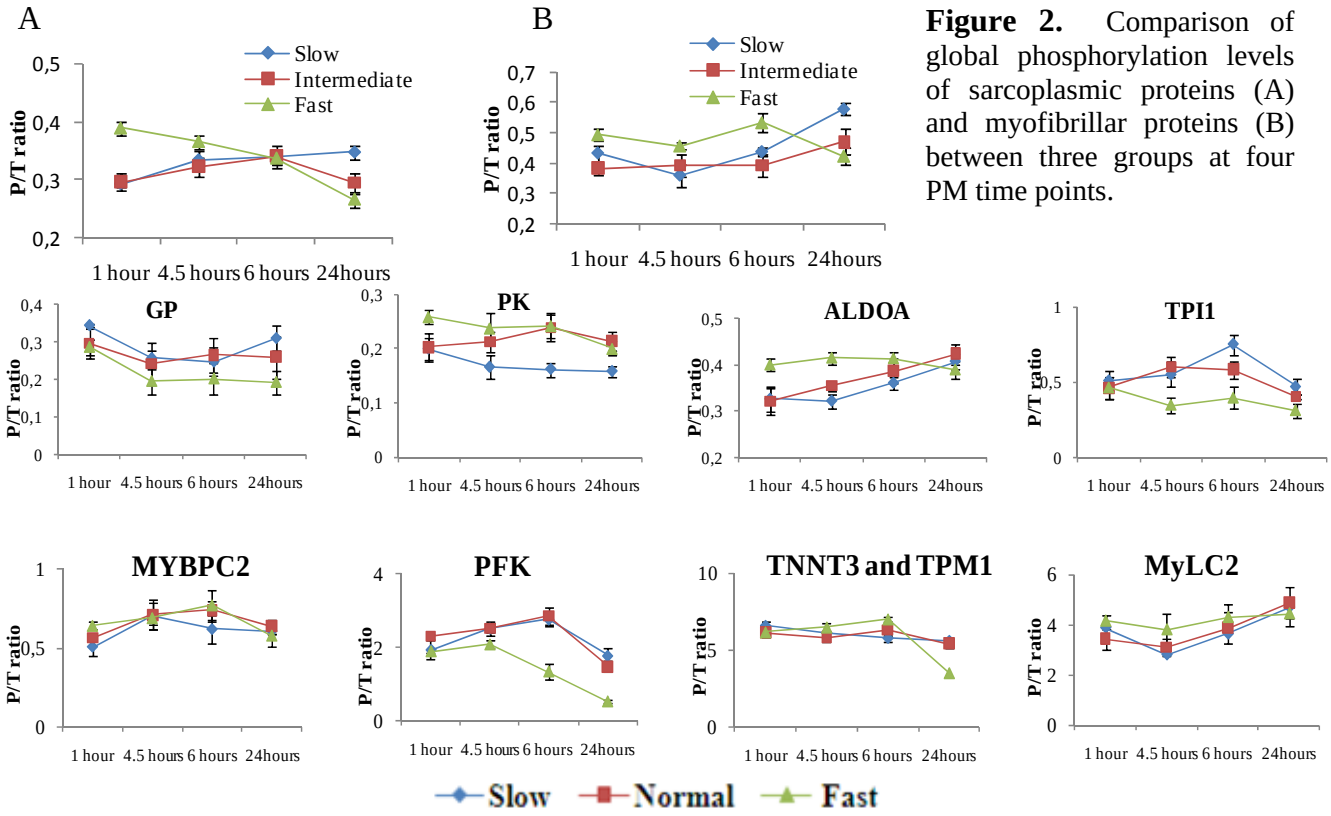

Figure 2. Comparison of global phosphorylation levels of sarcoplasmic proteins (A) and myofibrillar proteins (B) between three groups at four PM time points.

Figure 3. Change patterns of protein phosphorylation levels of individual phosphoproteins. glycogen phosphorylase (GP), Pyruvate kinase (PK), Fructose-bisphosphate aldolase C-A (ALDOA) and Triosephosphate isomerase 1 (TPI1) were identified from sarcoplasmic bands 7, 11, 15 and 20; Myosin binding protein C (MYBPC2), Muscle phosphofructokinase (PFK), Troponin T and Tropomyosin alpha-1 (TNNT3 and TPM1) and Myosin regulatory light chain 2 (MyLC2) were identified from myofibrillar bands 5, 9, 13 and 19.

Table 1. Effects of Time and pH on band P/T ratio

Sarcoplasmic Band No.	p values of effects			Myofibrillar Band No.	p values of effects		
	2-way pH*Time	Time	pH		2-way pH*Time	Time	pH
1	0.360	0.809	0.195	**1**	0.99	0.399	0.107
2	<0.001 ***	0.007 **	0.404	**2**	0.212	0.001 **	0.368
3	0.142	0.119	0.596	**3**	0.847	<0.001 ***	1
4	0.009**	0.016 *	1	**4**	0.955	0.197	0.921
5	<0.001 ***	0.036 *	0.987	**5**	0.036 *	<0.001 ***	0.813
6	<0.001 ***	0.327	0.960	**6**	0.429	<0.001 ***	0.859
7	0.136	0.010 *	0.799	**7**	0.221	0.895	1
8	0.0186 *	0.023 *	0.703	**8**	0.110	0.551	0.921
9	<0.001 ***	0.016*	0.293	**9**	0.014*	<0.001 ***	0.152
10	<0.001 ***	0.037 *	0.663	**10**	0. 311	<0.001 ***	0.676
11	0.004 **	0.102	0.677	**11**	0.150	0.294	1
12	0.018 *	0.452	0.762	**12**	0.396	0.193	1
13	0.480	0.340	1	**13**	0.567	<0.001 ***	0.945
14	0.960	<0.001 ***	1	**14**	0.843	<0.001 ***	0.441
15	0.001 **	<0.001 ***	0.720	**15**	0.159	0.063	0.435
16	0.886	0.061	1	**16**	0.316	0.494	0.609
17	0.349	<0.001 ***	0.868	**17**	0.158	0.346	0.499
18	0.991	0.073	0.676	**18**	0.153	0.456	0.786
19	0.640	0.278	0.740	**19**	0.655	<0.001 ***	0.739
20	0.018*	0.005 **	0.278				
21	0.843	0.001**	0.110				
22	0.022 *	<0.001 ***	0.630				
23	0.304	0.061	0.725				

Significance level: 0 '***' 0.001 '**' 0.01 '*' 0.05