Food Chemistry 134 (2012) 1999–2006

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Changes in phosphorylation of myofibrillar proteins during *postmortem* development of porcine muscle

Honggang Huang [a], Martin R. Larsen [b], René Lametsch [a,*]

[a] Department of Food Science, Faculty of Life Sciences, University of Copenhagen, DK-1958, Frederiksberg, Denmark
[b] Department of Biochemistry and Molecular Biology, University of Southern Denmark, Denmark

ARTICLE INFO

Article history:
Received 27 September 2011
Received in revised form 16 March 2012
Accepted 29 March 2012
Available online 10 April 2012

Keywords:
Protein phosphorylation
Porcine muscle
Myofibrillar proteins
Rigor mortis
Postmortem metabolism

ABSTRACT

A gel-based phosphoproteomic study was performed to investigate the *postmortem* (PM) changes in protein phosphorylation of the myofibrillar proteins in three groups of pigs with different pH decline rates, from PM 1 to 24 h. The global phosphorylation level in the group with a fast pH decline rate was higher than that in the slow and intermediate groups at early PM time, but became the lowest at 24 h. The protein phosphorylation level of seven individual protein bands was only significantly ($p < 0.05$) affected by PM time, and two protein bands were subjected to a synergy effect between PM time and pH decline rate. A total of 35 non-redundant highly abundant proteins were identified from 19 protein bands; most of the identified proteins were sarcomeric function-related proteins. Myosin-binding protein C, troponin T, tropomyosin and myosin regulatory light chain 2 were identified in the highly phosphorylated protein bands with the highest scores. The results indicate that the phosphorylation pattern of myofibrillar proteins in PM muscle is mainly changed with PM time, but only to a minor extent influenced by the rate of pH decline, suggesting that the phosphorylation of myofibrillar proteins may be related to the meat *rigor mortis* and quality development.

1. Introduction

Myofibrillar proteins are the largest protein class in skeletal muscle, and account for 55–60% of total protein content by weight. Myofibrillar proteins are responsible for the contractile properties of the muscle and for most of the functional and culinary properties of muscle and meat (Lee, Joo, & Ryu, 2010). During the conversion of muscle to meat in the early PM period, the exhaustion of ATP and the release of Ca^{2+} from the sarcoplasmic reticulum make the muscle continuously contracted but unable to return to relaxation; this process results in *rigor mortis* development. A high consumption of ATP causes a greater metabolic flux through the glycolytic pathway and leads to a rapid pH decline rate in PM muscle which accelerates and reduces the overall time of *rigor mortis* (Dickens & Lyon, 1995; Savell, Mueller, & Baird, 2005). The shortened sarcomere length at *rigor mortis* has a great influence on meat tenderness and other meat qualities (Koohmaraie, 1996; Tornberg, 1996). Myofibrillar proteins regulating muscle contraction could be involved in the *rigor mortis* development and therefore affect the meat quality. In-depth characterisation of myofibrillar proteins in PM muscle would benefit the understanding of meat quality development.

Reversible protein phosphorylation is one of the most widespread regulatory mechanisms in nature. Phosphorylation and dephosphorylation of proteins regulate critical biological processes, including metabolism, signalling transduction, proliferation and differentiation (Graves & Krebs, 1999; Hunter, 2000). In recent years, with the development of phosphoproteomic methods, protein phosphorylation has been deeply studied in various muscle samples (Gannon, Staunton, O'Connell, Doran, & Ohlendieck, 2008; Hojlund et al., 2009; Hou et al., 2010), and a large portion of the phosphoproteins identified in these studies were myofibrillar proteins. Phosphorylation of myofibrillar proteins plays essential roles in physiological functions in the living muscle. Phosphorylation of proteins that regulate functional activity of actomyosin plays essential roles in smooth muscle contraction (Vorotnikov, Krymsky, & Shirinsky, 2002); phosphorylation of cardiac isoforms of titin and filament proteins were involved in the modulation of sarcomeric function in cardiac muscle (Fukuda, Granzier, Ishiwata, & Kurihara, 2008; Solaro, 2008), and phosphorylated myomesin and myosin regulatory light chain 2 (MyLC2) were reported to have effects on skeletal muscle contraction and protein interaction (Obermann, Gautel, Weber, & Furst, 1997; Ryder, Lau, Kamm, & Stull, 2007; Zhi et al., 2005). In PM muscle, protein phosphorylation is known to play important roles in meat quality development by regulating the activities of glycolytic enzymes. The activities of glycogen phosphorylase (GP) (Johnson, 1992), pyruvate kinase

* Corresponding author. Tel.: +45 3533 3483; fax: +45 3533 3341.
E-mail address: rla@life.ku.dk (R. Lametsch).

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.03.132

(PK) (Schwagele, Haschke, Honikel, & Krauss, 1996), phosphofructokinase (PFK) (Cai, Callaci, Luther, & Lee, 1997) and AMP-activated protein kinase (AMPK) (Shen & Du, 2005) are influenced by their phosphorylation status; it has also been reported that MyLC2 became doubly phosphorylated during rigor formation in bovine longissimus (Muroya, Ohnishi-Kameyama, Oe, Nakajima, Shibata, & Chikuni, 2007).

Recently, we performed the gel-based phosphoproteomic analysis of sarcoplasmic proteins from PM porcine muscle and revealed that the phosphorylation patterns of several glyco-metabolism-related enzymes were affected by PM pH decline rate and time (Huang, Larsen, Karlsson, Pomponio, Costa, & Lametsch, 2011). However, the knowledge of protein phosphorylation in myofibrillar proteins of PM porcine muscle is still unclear. In this study, the combination of one dimensional gel electrophoresis (1-DE), coupled with a Pro-Q Diamond-Sypro Ruby staining and tandem mass spectrometry (MS/MS) strategy, was employed to investigate the changes in protein phosphorylation during *postmortem* development and to evaluate the effects of PM time and different pH decline rates on the protein phosphorylation levels of porcine myofibrillar proteins.

2. Materials and methods

2.1. Animal information

The animal samples used in this work were collected from our previous work (Pomponio, Ertbjerg, Karlsson, Costa, & Lametsch, 2010). Eighty pigs from an Italian Duroc× (Landrace × Large White) cross-bred pig population with an average live weight of 162 ± 12 kg were slaughtered. The pH value was determined at 1 h (pH 1) and 3 h *postmortem* (pH 3) at the 7th rib of the right side of the carcass. Then, 30 pigs were selected and divided into three groups (10 in each group) according to their 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). Animals with extremely fast pH decline (PSE like) were excluded (pH 1 h <5.7). Muscle samples at the 8th rib were taken at 1, 4.5, 6 and 24 h PM and stored at −80 °C. For our experiments, five pigs were randomly selected from each pH group. For each animal, the samples from the above-mentioned 4 time points were analysed.

2.2. Protein extraction

One gramme of frozen muscle sample was minced and homogenised on ice in 6 ml of ice–cold homogenising buffer containing 100 mM Tris pH 8.3, 10 mM DTT, complete protease inhibitor (Roche, Hvidovre, Denmark, one tablet per 50 ml) and phosphatase inhibitor PhosStop (Roche, Hvidovre, Denmark, two tablets per 50 ml).Then the samples were centrifuged at 4 °C for 20 min at 25,000 × *g*; the supernatant was carefully collected for sarcoplasmic protein analysis. After removing all the visible supernatant, the pellet (mainly containing the myofibrillar proteins) was dissolved in 25 ml of 5% SDS buffer by homogenisation at 9,500 rpm for 30 s and incubated in a water bath for 30 min at 80 °C, then aliquoted and stored at −80 °C until used. The resulting pellet solution was used for myofibrillar protein analysis. The protein concentration was determined using the BCA assay (Pierce Chemical Company, Rockford, IL).

2.3. Gel electrophoresis and image analysis

For one-dimensional gel electrophoresis, the volume of each muscle supernatant containing 50 microgrammes of protein was well mixed with 25 μl of NuPAGE LDS Sample Buffer (4×) (Invitrogen, Denmark), 10 μl of NuPAGE Reducing Agent (10×) (Invitrogen, Denmark), and deionized water together to 100 μl. The mixture was heated for 10 min at 70 °C. An aliquot of 10 μl of mixed sample buffer was loaded on one well of a 4–12% NuPAGE Novex 15 wells, Bis–Tris Mini Gel (Invitrogen, Denmark). The gels were run in NuPAGE MES running buffer at 200 V constant voltage until the blue dye front disappeared from the bottom of the gel. Protein samples at four time points of three animals from slow, intermediate and fast pH decline groups were loaded onto the same gel, and made in triplicate. A total of 15 gels were used for the analysis. The detection of the phosphoproteins was performed using Pro-Q Diamond according to the manufacturer's instruction (Invitrogen, Denmark). Fluorescently-labelled proteins were visualised using the Typhoon Triovariable mode imager system from GE Healthcare. Images were scanned on Cy3 channel (excitation 532/25 nm, emission 580/25 nm, 500 V) at 200 mm resolution. For total proteins, the same gels were stained overnight in 50 ml of SYPRO Ruby dye as by manufacturer's instruction (Invitrogen, Denmark), and scanned on SYPRO Ruby channel (excitation 532/25 nm, emission 610/25 nm, 500 V) at 200 mm resolution. To ensure the specificity of our staining method, the broad range SDS–PAGE molecular weight Marker (Bio-Rad), containing a mixture of phosphorylated and nonphosphorylated proteins, was used as the standard control. Finally, the gels were stained with colloidal Coomassie Brilliant Blue R-250 and imaged to visualise the protein locations. Images from both Pro-Q Diamond and SYPRO Ruby staining were subjected to ratiometric analysis to compare the protein phosphorylation changes. The intensities of the same protein band from Pro-Q Diamond and SYPRO Ruby gels were determined from a rectangular area containing the entire band, along each sample lane, using TotalLab TL120 software package (TotalLab, Germany). The protein phosphorylation level was evaluated by determining the ratio of the intensity of phosphoprotein (P) in each band in the Pro-Q Diamond image to its intensity of total protein (T) in SYPRO Ruby image (P/T ratio). The global P/T ratio of each sample was calculated by measuring the fluorescence intensity over the length of a lane that included all bands on differently stained gels (Huang et al., 2011; Schulenberg, Aggeler, Beechem, Capaldi, & Patton, 2003; Silverman-Gavrila, Lu, Prashad, Nejatbakhsh, Charlton, & Feng, 2009).

2.4. In-gel trypsin digestion and protein identification by MALDI–TOF–TOF–MS/MS

Protein bands were excised from Coomassie-stained gels and subjected to in-gel trypsin digestion. The excised gel plugs were washed three times for 15 min each in 30 μl of 50% acetonitrile/50 mM NH_4CO_3, pH 7.8, and then shrunk in 30 μl of 100% acetonitrile. The proteins were reduced with 30 μl of 50 mM NH_4CO_3, 100 mM DTT for 45 min at 56 °C, and alkylated with 30 μl of 50 mM NH_4CO_3, 150 mM iodoacetamide for 45 min at room temperature in the dark. Then the gel piece was washed with 30 μl of 50% acetonitrile/50 mM NH_4CO_3, pH 7.8, and dehydrated with 30 μl of 100% acetonitrile. 15 μl of trypsin solution (sequencing grade, Roche, Mannheim, Germany) (10 ng/μl of trypsin dissolved in 50 mM of NH_4CO_3, pH 7.8) was added to the dry gel pieces and incubated on ice for 1 h, then the unabsorbed buffer was removed and replaced with 30 μl of 50 mM NH_4CO_3, pH 7.8. The proteins were digested overnight at 37 °C. Columns consisting of 0.2 μl of Poros reversed phase R2 material (20 μm bead size, PerSeptive Biosystems, Framingham, MA) were packed in a constricted GeLoader tip (Eppendorf, Hamburg, Germany) in advance. 20 μl of the digested peptide were acidified by trifluoroacetic acid (TFA), loaded onto the column, and washed with 20 μl of 0.1% TFA. Finally, the peptides were eluted with 0.5 μl of matrix solution