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# Phosphoproteome analysis of sarcoplasmic and myofibrillar proteins in bovine *longissimus* muscle in response to postmortem electrical stimulation

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ABSTRACT

Protein phosphorylation changes of the sarcoplasmic and myofibrillar proteins in beef *longissimus* muscle in response to electrical stimulation (ES) was investigated. Sarcoplasmic and myofibrillar proteins purified from muscle samples taken at 0, 3 and 10 h after ES were separated on SDS–PAGE and stained with phosphorous and protein specific stains. There was a significant effect of ES on phosphorylation of total sarcoplasmic and myofibrillar proteins ($P < 0.05$). However, although there an instant effect of ES on the phosphorylation level of the myofibrillar proteins, the ES effect on the sarcoplasmic proteins ($P < 0.05$) was first observed after 3 h. Several protein bands were analyzed by LC–MS/MS, revealing that the major glycolytic proteins, including glycogen debranching enzyme, glycogen phosphorylase and 6-phosphofructokinase probably are affected by ES together with different heat shock proteins. This work gives an insight into the regulation of the glycolytic enzymes and muscle contraction on application of electrical stimulation.

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## 1. Introduction

Electrical stimulation (ES) after dressing has been shown to accelerate the postmortem tenderization of beef muscles effectively (Hollung et al., 2007; Li et al., 2012), probably by inducing faster energy depletion and pH decline, earlier activation of μ-calpain, earlier release of lysosomal enzymes, and the formation of contraction nodes (Li et al., 2012). In a proteomic study, ES induced significant changes in abundance of 10 proteins at 1 h after ES, 13 proteins at 24 h after ES and five proteins at both 1 and 24 h (Bjarnadóttir, Hollung, Høy, & Veiseth-Kent, 2011).

In terms of molecular changes after slaughter, phosphorylation of glycolytic enzymes plays an important role in regulating their activities, as a result of muscle pH decline rate and meat quality development (D'Alessandro, Rinalducci, et al., 2012; Huang et al., 2011). Phosphorylation of myofibrillar proteins may mediate the progress of rigor mortis, and phosphorylation of heat shock proteins (HSPs) may change their functions via their affinity for targets (D'Alessandro, Marrocco, et al., 2012; Muroya et al., 2007). However, the importance of protein phosphorylation for the conversion of muscle to meat, in particular for application of ES, has been less concerned.

In a previous study, the authors found that ES changed the phosphorylation levels of 1 pyruvate kinase isoform, 3 β-enolase isoforms, 2 creatine kinase M chain isoforms and 2 fructose-bisphosphate aldolase C-A isoforms at 3 h postmortem based on two-dimensional electrophoresis and ProQ-staining (Li et al., 2012). The objective of the present study was further to investigate phosphorylation of beef sarcoplasmic and myofibrillar proteins at early postmortem time in response to electrical stimulation and postmortem time.

## 2. Materials and methods

### 2.1. Sampling

Nine Swedish red cattle, including 4 young bulls (up to 18 mo.) and 5 steers (24–36 mo), were slaughtered commercially according to Council Regulation (EC) No. 1099/2009. Just before chilling (about 30 min after stunning), the left sides were electrically stimulated by an electrical stimulation apparatus (Micro Technic

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and DMRI, Denmark). The stimulation applied was a square-wave and direct current, with pulses of 5 ms duration and 65 ms pause interval and peak voltage of 80 V for 35 s. The right sides were not stimulated as control.

*Longissimus lumborum* muscles were removed from both carcasses immediately after electrical stimulation and placed at approximately 10 °C for 12 h to avoid cold shortening. About 10 g of muscle was sampled at 0 h, 3 h and 10 h after stimulation. The samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C for further analyses.

### *2.2. Protein extraction*

Duplicate of 1 g muscle tissue were homogenized (Ultra Turrax Mixer T 25, IKA labortechik, Königswinter Germany) in 6 ml of cold buffer (100 mM Tris, pH 8.3, Roche Complete (one tablet per 50 mL), Roche PhosStop (one tablet per 25 mL)) for 2 × 30 s at 9500 rpm and 2 × 30 s at 13,500 rpm with 30 s cooling between bursts. The homogenates were centrifuged for 20 min at 15,000×*g* at 4 °C. The supernatant (sarcoplasmic fraction) was transferred to one 15 mL centrifuge tube and stored at −20 °C for less than 7 days. The pellet was dissolved in 25 mL 5% SDS solution (60 °C) and then transferred to 50 mL test tubes (myofibrillar fraction). The myofibrillar fraction was re-homogenized at 9500 rpm for 30 s and heated at 80 °C for 20 min, and then stored at 4 °C. The protein concentration of sarcoplasmic and myofibrillar fraction was determined at 280 nm (Helios Omega UV–VIS, Thermo Scientific, Waltham, MA USA) with one unit of absorbance representing a concentration of 1 mg protein/mL.

### *2.3. SDS–PAGE*

Appropriate volumes of sarcoplasmic or myofibrillar samples were mixed with 12.5 μL NuPAGE LDS sample buffer (4×) and 5 μL NuPAGE reducing sample agent (10×) and made up to a total volume of 50 μL with ultrapure water. The final protein concentration was 0.75 μg/μL for sarcoplasmic fraction and 0.3 μg/μL for myofibrillar fraction. The samples were heated at 70 °C for 10 min. Ten microliters of samples were loaded in triplicate into the wells of NuPAGE® Novex® Bis–Tris mini gels (Invitrogen, Grand Island, NY, USA). The gels were run in a total 800 mL of 1 × NuPAGE SDS running buffer at 200 V till the blue dye front disappeared. For sarcoplasmic samples, 500 μL NuPAGE antioxidant was added in the running buffer in the inner chamber.

### *2.4. Staining and imaging for phosphoproteins and total proteins*

The gels were stained by ProQ Diamond stain for phosphoproteins, then by SYPRO Ruby stain and finally by Coomassie stain for total proteins.

Before staining, gels were fixed overnight in 120 mL of fixative [50% (v/v) ethanol, 10% (v/v) acetic acid] with a change at 0.5 h, and then washed for 10 min in 120 mL of ultrapure water with 3 changes. After rinsing, gels were incubated in 80 mL of phosphor-specific ProQ Diamond stain (Invitrogen Molecular Probes, Eugene, OR, USA) for 90 min in the dark and then destained for 30 min in the dark in 120 mL of destaining solution (0.05 M sodium acetate, pH 4.0, 20% acetonitrile) with two changes. After three washes in ultrapure water for totally 20 min, the gels were scanned with a Typhoon Triovariable scanner (GE Healthcare, Uppsala, Sweden) with the fluorescence mode under a filter of excitation 532/25 nm, emission 580/25 nm, and voltage 500 V with normal sensitivity.

After scanning, the same gels were stained in the dark overnight in 80 mL of SYPRO Ruby stain (Invitrogen Molecular Probes, Eugene, OR, USA). Following twice 30 min rinse in 10% methanol and 7% acetic acid and one 10 min rinse in ultrapure water, the gels were visualized with the Typhoon scanner with the fluorescence mode under a filter of 532 nm, 610(30) band-pass, 532-nm laser, and 470 V with normal sensitivity.

Finally, the gels were stained with colloidal Coomassie brilliant blue R-250 just to localize protein bands on gels for further identification.

### *2.5. Image analysis*

Images from ProQ Diamond stained and SYPRO Ruby stained gels were further analyzed and compared. The band intensities on SDS–PAGE gels were quantified with the Phoretix 1D image analysis software (version 1, Nonlinear Dynamics Ltd., FL, USA).

### *2.6. Protein identification*

A total of 24 bands of interest from SDS–PAGE gels were identified by using LC–MS/MS. In brief, each protein gel band was destained, cleaned, and digested in-gel with sequencing grade modified trypsin (Promega, Madison, WI, USA). The resulted peptides mixture was analyzed by a LC–MS/MS system (LCQ DECA XP Plus, Thermo, Palo Alto, CA, USA), in which a high performance liquid chromatography (HPLC) with a 75 μm inner diameter reverse phase C18 column was on-line coupled with a Quadrupole ion trap mass spectrometer. The mass spectrometric data acquired with the Xcalibur 2.0 software (Thermo, Palo Alto, CA, USA) under the centroid mode by minimum MS signal for Precursor-ion of $5 \times 10^4$ counts were used to search non-redundant protein database (NCBI's GenBank) with ProtTech's proprietary software suite ProtQuest (Norristown, PA, USA). The main processing parameters including signal-to-noise ratio of not less than 5, missed cleavages of no more than 5 and modifications of Cys+57. The output from the database search was manually validated before reporting.

### *2.7. Statistical analyses*

The phosphorylation level of individual proteins band was evaluated by a ratio of its intensity in ProQ Diamond stained gel to its corresponding intensity in SYPRO Ruby stained gel. The total phosphorylation level was expressed as the sum of phosphorylation levels of individual proteins bands on the same gel.

The effects of ES and postmortem time on band intensity were evaluated using a mixed ANOVA (analysis of variance) model in which animal was set as random effect and ES and postmortem time were set as fixed effect. The differences of least squares means between ES and control groups, and among three time points were evaluated after Bonferroni adjustment. If the interaction was significant, the differences of least squares means were further compared among treatment × time subgroups. These analyses were performed under the program SAS 9.2 (SAS Institute Inc., Cary, NC, USA, 2008).

## 3. Results and discussion

### *3.1. Phosphorylation of sarcoplasmic proteins in response to electrical stimulation and time*

A total of 26 protein bands were detected on the SDS–PAGE gels containing the sarcoplasmic proteins (Fig. 1a). The phosphorylation of total sarcoplasmic proteins, calculated as the sum of *P*/*S* ratios of 26 bands, showed a slight increase with postmortem time (Fig. 1b) for both the ES and control muscles. There were a larger increase in the ES samples compared with the control ($P < 0.05$). It has previously been reported that pigs with a moderate or slow

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